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Pathogenic Fungi in Ferns and Angiosperms: A Comparative Study

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Abstract.—This study used existing databases to test the hypothesis that fern species harbor fewer pathogen species than angiosperm species. Analysis was limited to fungal pathogens because of their visibility and to herbaceous perennial dicots (forbs) because they have a similar growth form to ferns. From complete listings in the United States Department of Agriculture plant-fungal database, the number of pathogen species recorded on 200 randomly chosen ferns and herbaceous perennial dicot species were assessed. To control for differences in study effort, the number of citations to these species in the Web of Science was determined. The results showed that the major predictor of the number of fungal pathogen species known to occur on a plant species was study effort, but after controlling for this, the likelihood of a fern species being recorded as having a fungal pathogen species was much less than that for a forb. When pathogens were present, there were approximately 50% fewer pathogen species recorded on fern species than on forb species. This pattern is present even though fern species were cited on average more often than forb species, and it is consistent with impressions in the literature from studies in other parts of the world. Testable hypotheses to explain this difference are evaluated in the context of evolutionary processes leading to variation for pathogen incidence in different phylogenetic lineages, but the physiological or molecular processes that determine the higher resistance of ferns to fungal pathogens remain unknown.

Key Words.—disease resistance, parasite species richness, Uredinales, Ustilaginales, Microbotryales

This study set out to test the simple hypothesis that ferns harbor fewer fungal pathogens than flowering plants. This hypothesis was initially driven by my own casual natural history observations and readings, suggesting that pathogens are rare in both ferns (Page, 2002) and mosses (Berkeley, 1862; Davey and Currah, 2006). This is especially surprising as ferns and mosses often grow in dense mono-specific stands in moist, shaded areas where infection, especially by fungi, might be expected to be common. Similar conjectures have been made by other authors (Saxena and Harinder, 2004), and while it is generally accepted that the most common fungal pathogens of the ferns are the rust fungi (Uredinales), smut fungi (Ustilaginales, Microbotryales) have been considered to be rare or absent (Helfer, 2006; Oberwinkler, 1994). This can also be inferred by the absence of ferns as hosts in comprehensive monographs on smut fungi (Vanky, 1994; Woods *et al.*, 2018).

Whether ferns have fewer pathogens than flowering plants is a question that is not just a matter of curiosity but impinges on understanding fern evolution, their ecology, and may be important from an applied perspective. There is increasing recognition that pathogens play an important role in the ecology and evolution of natural populations of plants and animals (Gilbert, 2002), influencing their extinction risk (DeCastro and Bolker, 2005), their nutrient cycling and food web structure (Lafferty, Dobson, and Kuris, 2006), as well as other processes such the evolution of sex (Lively, 2010) or speciation (Bomblies and Weigel, 2007). However, in ferns the major focus has been on their phylogenetic relationships, chromosome evolution, and mycorrhizae or endophytes as fungal symbiotic partners (Pressel *et al.*, 2016; Read *et al.*, 2000). There have been some previous reviews of fungal pathogens on ferns both in the US and worldwide (Helfer, 2006; Stevenson, 1945), but they have been brief and focused on documenting the major types of pathogens recorded on ferns, and have not involved a quantitative assessment of this diversity in a comparison with other types of plant.

Comparative studies of pathogen occurrence can be problematic, not only because of their taxonomic and ecological diversity, but also because data on pathogens in natural populations (as opposed to humans, crops, and domesticated animals) is still sparse and fragmentary. It is now widely appreciated that a major source of 'noise' in analyzing such data is that the number of pathogens recorded on a particular host species is highly correlated with the study effort devoted to that species (Antonovics and Hayden, 2020; Williams, Antonovics, and Rolff, 2011) and this is a characteristic feature of all disease databases (Nunn and Altizer, 2006; Walther *et al.*, 1995).

In this study, I narrow the question to the specific consideration of fungal pathogens of ferns in the United States. Fungal pathogens generally produce highly visible lesions, and are readily identifiable at least to genus based on sporangial and spore characteristics; viral and bacterial pathogens are generally far more cryptic and even less well understood taxonomically. There are substantial data on the world-wide occurrence of fungal pathogens on ferns and higher plants in the USDA databases. These data (and earlier related sources) have been used in previous assessments of the occurrence of fungal pathogens on ferns both in the US and worldwide (Helfer, 2006; Stevenson, 1945).

After correcting for study effort, the results show that fungal pathogens are less likely to be recorded as present on fern species relative to herbaceous perennial angiosperms, and that if found, fewer pathogen species are present. I discuss possible reasons for this difference, and its implications.

MATERIALS AND METHODS

I narrowed the data to only consider fungal pathogens found on dicot herbaceous perennials (*i.e.*, forbs), a growth habit largely reflecting the life-form of most ferns. Shrubs and trees were excluded, not only because of their different life form, but because in the USDA plant-fungal data base it is often not clear whether the fungi that are recorded are attacking woody tissue, and therefore largely saprophytic rather than parasitic; the earlier editions of the USDA database explicitly included pathogens found on wood-products. The list of potential host plants was obtained from the PLANTS database made available by the Natural Resources Conservation Service of the USDA (https://plants.sc.egov.usda.gov/java/). The list of fern species was obtained by using the Advanced Search Download tab, with the options ‘PLANTS Floristic Area or Not=North America’ and ‘Category=Fern’. The broad option ‘North America’ was used because some trial searches indicated that the sub-option ‘Lower 48 U.S. States’ did not return some expected species. Checking the box ‘Download text file without formatted display’ allowed download of the species list, from which hybrid ferns were excluded, leaving 370 species.

The list of Angiosperms was obtained by Advanced Search in the same database, using the options ‘PLANTS Floristic Area or Not=North America’, ‘Category=Dicot’ and ‘Duration=perennial’ with the adjacent ‘display’ box checked, and ‘Growth Habit=Forb/herb’ with the adjacent ‘display’ box checked. As above, the list was downloaded and only those species with a perennial designation plus a forb/herb designation were included (species with double classifications such as biennial/perennial, shrub/forb were excluded). A total of 5,431 species were returned. Throughout, the option ‘Scientific Name, include=Accepted Names Only’ was used, and no attempt was made to revise any taxonomy.

From each of these lists, 200 species of ferns and 200 species of forbs were chosen at random, and without regard to any other species characteristics. These two sets of fern and perennial species were then assessed for ‘study effort’ by finding the number of search results in the Web of Science database (http://apps.webofknowledge.com/WOS_GeneralSearch_input.do?product=WOS&search_mode=GeneralSearch&SID=8DO4ciNdr11xcSx5AVL&preferencesSaved=), using the option “Select a database=All databases’. The species binomial was entered without quotes and under ‘Topic’ with no restriction as to geography, and spanning the years 1864-2019. Study effort on a species was simply the number of times that species had been cited as a ‘Topic’. The search results returned publications that have the species name in the title, abstract, or key words. The Web of Science does not always take into account nomenclatural changes/synonyms in species names, and all species names entries were as in the USDA plants database.

To assess the number of fungal pathogen species recorded on each host species (sometimes termed the ‘parasite species richness’; Nunn *et al.*, 2003), the USDA Agricultural Research Service database was used (https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm) under the tab ‘Fungus-Host’. The searches were restricted to ‘Available Localities=United States-All’. Following display of the pathogens recorded for each fern and perennial herb species, the total number (if any) were counted after removal of displayed synonyms and of pathogens with only a genus name but for which previously a genus and species name had also been given.

The statistical analysis was carried out in two steps using the R package (R Core Team, 2018). In the first step, to assess if the frequency with which at least one pathogen species was detected depended on the study effort, a logistic regression of presence (1) and absence (0) of a pathogen on a species vs. number of citations for that species was estimated. Using the function ‘glm’, a test for an interaction was carried out with the model (number of pathogens) ~ (type) * (number of citations), with a binomial link, and where type = fern or herb and number of citations (x) was log10 (x+1) transformed (a few species had zero citations in the Web of Science even though listed in the USDA database). As the interaction term was not significant, I tested for an effect of host type and if number of citations affected the likelihood that a pathogen would be recorded using the model (number of pathogens) ~ (type) + (number of citations)

The second step was applied only to those host species where one or more pathogen species had been recorded. To assess if the number of pathogens detected differed among host types (fern or herb), the same model as above was used, except with a Gaussian link (assuming normally distributed error) and using log10 (x) transformed numbers for pathogen and host species because there were no zero values.

RESULTS

The probability of detecting a pathogen on a host increased significantly ($P<0.0001$) with the number of citations to that host; the interaction term in the logistic regression was not significant ($p<0.58$) indicating no difference between ferns and forbs in the slope of the regressions on a logistic scale. Pathogens were recorded as occurring significantly less frequently ($P=0.018$) on fern species than forb species after controlling for the search effort on each plant species (Fig. 1). The difference in means of the logistic regression was 0.678 (97.5% confidence intervals=0.764-1.43). Because logistic regression involves log transformation of the ratio of presence/absence, this difference is difficult to interpret straightforwardly, but inspection of Fig. 1 shows that at citation numbers of 100, pathogens are recorded on about half as many ferns as forbs.

In terms of total numbers for the 200 species of each type, there were 21,335 citations for ferns and 32 had at least one pathogen, whereas for herbs there were 14,095 citations and 41 had at least one pathogen. The slope of the relationship between pathogen occurrence and number of citations was 1.091 (0.25-97.5% confidence intervals=0.764-1.43), indicating that the log odds ratio of detecting a pathogen in the database increased by one for approximately every log10 number of citations.

Given that one or more pathogens were present, the number of pathogens found also increased significantly ($P<0.0005$) with the number of citations. The slope of the relationship between number of pathogens and number of citations was 0.198, indicating that for every ten-fold increase in the number of citations, there was a 1.57-fold (57%) increase in the number of pathogen species recorded. The interaction term was non-significant ($P<0.97$) indicating no difference between forbs and ferns in the slope of the regressions (on a log-log scale).

There were significantly ($P=0.0082$) fewer pathogens on ferns than on forbs (Fig. 2). The least square means when back transformed, indicated that there were 2.40 (confidence interval 1.73-3.33) pathogens predicted per citation in ferns and 4.28 (confidence interval 3.02-6.05) per citation in herbaceous dicots.

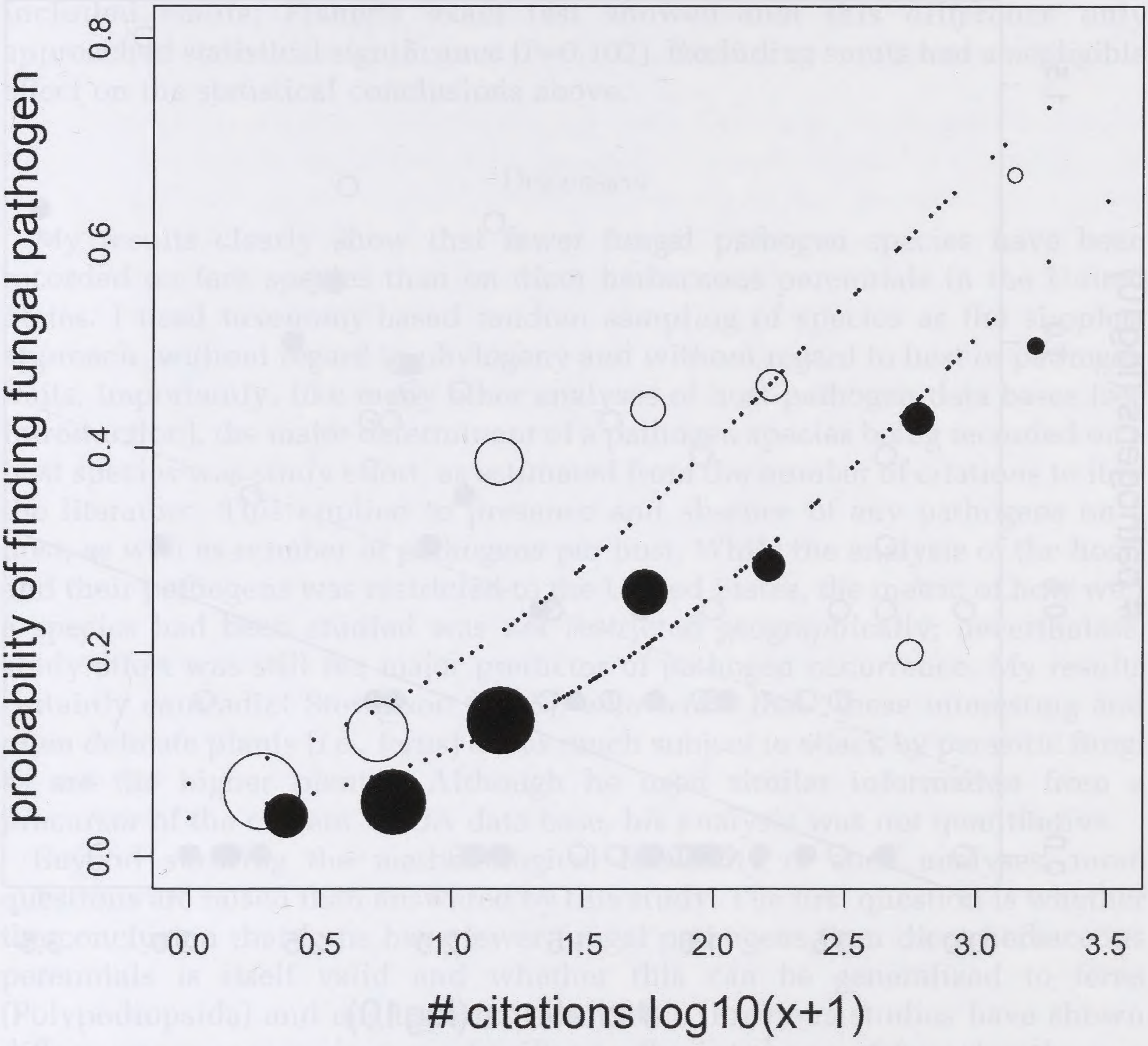

FIG. 1. The relationship between probability of detecting a fungal pathogen and number of citations to the host, back transformed from a linear logistic regression fit (see text). The points are predicted values from the logistic regression (upper series are herbaceous perennials). Open circles show observed probabilities, for forbs, and closed circles show probabilities for ferns when data are grouped into classes of 0-0.5, etc. on a log10 scale. Diameter of the dot is proportional to the number of citations in each of the classes.

In terms of actual numbers, for the 32 fern species with pathogens, there were 9,278 citations and 98 unique pathogen-host species combinations, whereas for herbs there were 6,353 citations and 203 unique pathogen-host species combinations.

I examined if there was evidence of phylogenetic signal in the data by comparing fungal incidence in monophyletic clades as determined by Pryer *et al.* 2004, and by Pteridophyte Phylogeny Group (2016). Because the occurrence of fungi was very sporadic (only 32 of the 200 fern species had records of any fungi, with uneven clade sampling), I used generalized linear models, again using log10(x+1) number of citations as a covariate, but assuming the number of fungi recorded were Poisson distributed (SAS Proc GLMMIX, model

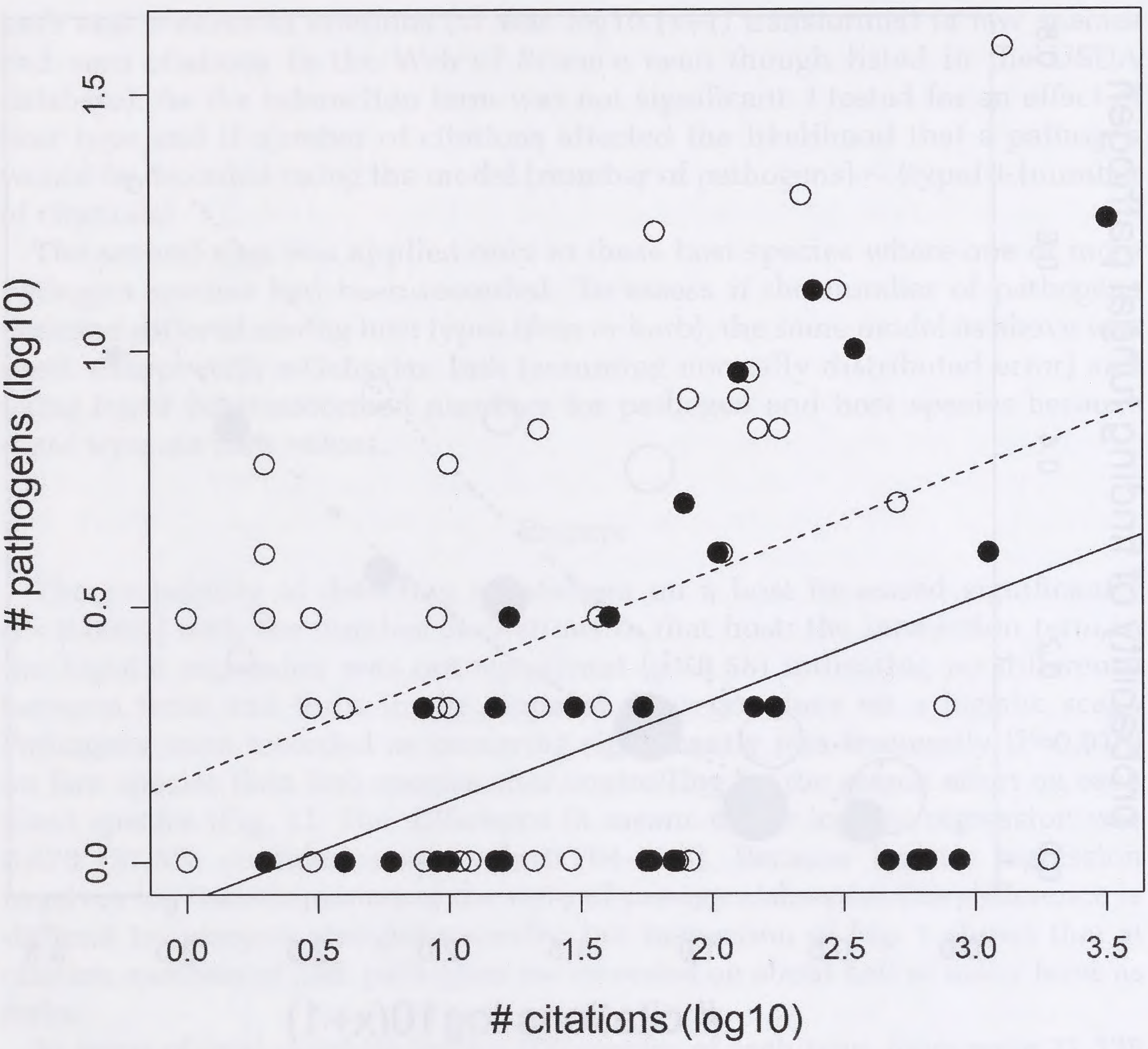

FIG. 2. The relationship between number of fungal pathogens detected on a host species and number of citations to that host (both on log10 scale). Black dots = ferns and open circles=forbs. Lines are linear regressions, solid=ferns, dashed = forbs.

fungi=citations order family(order), and correcting for overdispersion; SAS Institute 2016). The effect of orders ($F_{4,15}$=1.75, P <0.191) was not significant, but the effect of families was highly significant ($F_{15,179}$=2.25, P <0.0063). There were two sub-orders of the Polypodiales with more than one family in each, namely Aspleniinae and Polypodiinae, and a nested analysis of variance for an effect of sub-order and family showed no effect of suborder but also significant variation among families ($F_{9,89}$=2.29, P <0.023) within sub-order. However, variation among families in proportion of species with at least one record was not significant (P<0.70, using logit link). Genus effects within families were not significant.

Because there is evidence that ferns have few smut fungi (see Introduction) I also specifically searched the USDA plant-fungal database for 'Basidiomycota-Smuts' (Ustilaginales and Microbotryales). No smuts were recorded among the 98 pathogen-fern combinations while 7 of the 203 pathogen-forb combinations included smuts; Fisher's exact test showed that this difference only approached statistical significance (P=0.102). Excluding smuts had a negligible effect on the statistical conclusions above.

DISCUSSION

My results clearly show that fewer fungal pathogen species have been recorded on fern species than on dicot herbaceous perennials in the United States. I used taxonomy-based random sampling of species as the simplest approach, without regard to phylogeny and without regard to host or pathogen traits. Importantly, like many other analyses of host-pathogen data bases (see Introduction), the major determinant of a pathogen species being recorded on a host species was study effort, as estimated from the number of citations to it in the literature. This applied to presence and absence of any pathogens on a host, as well as number of pathogens per host. While the analysis of the hosts and their pathogens was restricted to the United States, the metric of how well a species had been studied was not restricted geographically; nevertheless, study effort was still the major predictor of pathogen occurrence. My results certainly contradict Stevenson (1945), who wrote that "these interesting and often delicate plants [*i.e.*, ferns] are as much subject to attack by parasitic fungi as are the higher plants." Although he used similar information from a precursor of the current USDA data base, his analysis was not quantitative.

Beyond showing the methodological feasibility of such analyses, more questions are raised than answered by this study. The first question is whether the conclusion that ferns have fewer fungal pathogens than dicot herbaceous perennials is itself valid and whether this can be generalized to ferns (Polypodiopsida) and angiosperms as a whole. Previous studies have shown differences among angiosperm families in the incidence of fungal pathogens (Antonovics and Hayden, 2020). In my data, there was also evidence of heterogeneity in fungal incidence among fern families, but the data were too sparse and unbalanced in taxon representation to do any detailed phylogenetic analysis. It therefore remains an open question if the difference I found between ferns and forbs is driven by the occurrence of particular taxa in the United States or if it is more general. An analysis with additional data from other regions would address this question, but prior qualitative assessments in other countries also argue that the patterns seen here may extend beyond the United States.

More than a century ago, the British mycologist Berkeley (1892, p.8) remarked that:

> "Nor are the vascular cryptogams, such as ferns, mosses, and liverworts without their own especial enemies, though these are fewer in number perhaps than in other organized beings."

Oberwinkler (1994, p.8), another outstanding mycologist, referring to plants in southern Germany, wrote (translated from German):

> "Powdery and downy mildews don't occur on ferns, nor do smut fungi, but there are several rust fungi."

In the Biological Flora of the British Isles (https://www.britishecologicalsociety.org/publications/journals/journal-of-ecology/biological-flora-database/), a series of thorough autecological reviews of species of British plants, seven fern species are considered. Five are noted as having no fungal pathogens, and *Dryopteris carthusiana* (Vill.) H. P. Fuchs as having only one. *Pteridium aquilinum* (L.) Kuhn, bracken, a well-studied species of economic importance because of its invasiveness and toxicity to cattle, is listed as having three pathogens on the prothalli, and seven on the adults, but Marrs and Watt (2006) conclude, "In short, *Pteridium*, like most ferns, is remarkably resistant to disease." Helfer (2006), in his review of worldwide pteridophyte pathogens in the USDA database, noted that rusts were absent in *Equisetum*, and that there was only one record from the lycophytes. None of the 20 most common genera of pathogens were smuts, downy, or powdery mildews. Hopefully the present study will stimulate studies of fungal and other pathogens of ferns beyond the United States to see if these patterns are robust to more thorough taxon sampling, especially in the tropics, and the extent to which they are dependent also on the type of fungal pathogen being considered.

The possibility that the observed pattern is the result of biases in the sampling prior to entry into the databases also cannot be excluded. For example, researchers on ferns may be less trained in or may have less interest in detecting fungal pathogens than researchers on herbaceous plants. Alternately, pathogens on ferns may be less visible than those on herbaceous perennials. However, these explanations are unlikely, as fern biologists have a 'strong-eye' for details of frond morphology and therefore would be expected to notice pathogen lesions if they were present. Moreover, analysis of the citation data showed that the average number of citations per species was significantly greater for ferns than for forbs (Fig. 3). The reason may be that in the United States there are far fewer fern species (370 in my listing) than forb species (5,428) and this, combined with a dedicated 'constituency' of fern biologists, has resulted in ferns being sampled more thoroughly than herbaceous perennials. The absence of any significant differences between ferns and forbs in the slopes of the regressions of pathogen occurrence vs. study effort indicates that the probability of detection 'per increase in study effort' is also similar in the two categories, albeit there is a large variation about these regressions.

The differing relative commercial relevance of ferns and flowering plants is also an unlikely contributory factor to their contrasting number of pathogens. Although crops have more pathogens than non-crop species (although not on a per citation basis; Antonovics and Hayden, 2020), no crops were present among the herbaceous perennial species. However, there were two horticulturally important species among the ferns, namely, *Nephrolepis exaltata* (L.) Schott, Boston fern, and *Cyrtomium falcatum* (L. f.) C. Presl, the holly fern; they had the highest proportion of pathogens per citation (13/245 and 9/127,

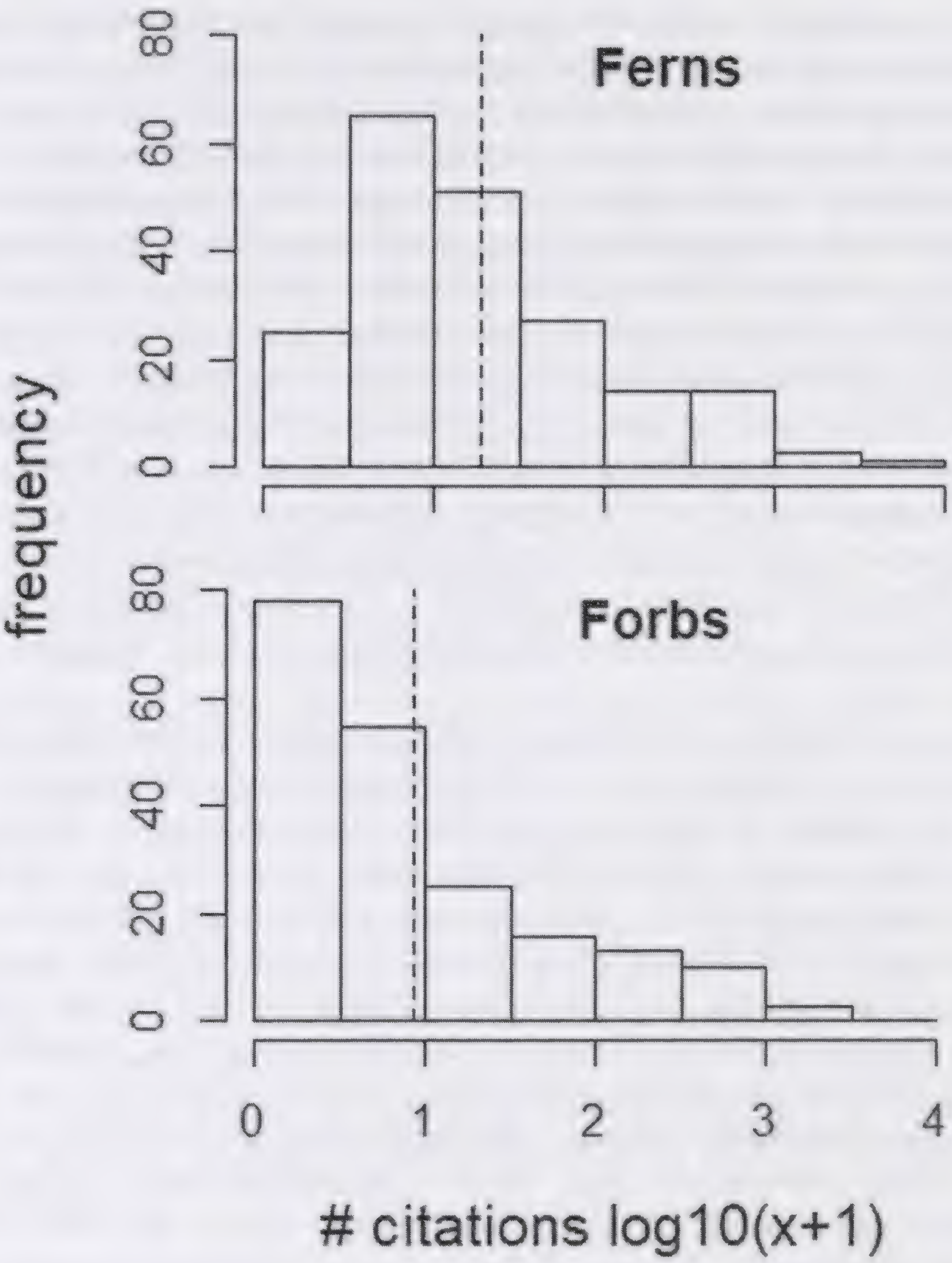

FIG. 3. Frequency of a given number of citations in the Web of Science for ferns and forbs. Dashed vertical lines indicate means. Number of citations are grouped on a log10 scale. The difference between the means is highly significant (P<0.001) both by heterogeneity chi-squared on classes in the histograms (=41.5, df=7) and by a t-test on log10(x+1).

respectively). The four most frequently cited ferns in my study sample were *Pteridium aquilinum* (3214 times), *Dryopteris filix-mas* (L.) Schott (1898), *Azolla filiculoides* Lam. (1167) and *Athyrium filix-femina* (L.) Roth (1133), while the most frequently cited forbs were *Silene vulgaris* (Moench) Garcke (1896), *Asclepias syriaca* L. (1295), *Lotus pedunculatus* Cav. (1156), and *Inula britannica* L. (827). The latter, a weedy species, was only introduced into the US in 1999 (Lehtonen, Schall, and Wager-Pagé, 2009).

It is possible to ask why ferns have fewer pathogens than forbs from either a direct mechanistic viewpoint (whether physiological or ecological) or from an evolutionary perspective (what are the evolutionary origins of the observed patterns). At present it is not known if ferns have different resistance mechanisms relative to angiosperms. Both ferns and angiosperms likely use secondary compounds with anti-fungal properties as well as mechanisms based on more specific molecular recognition. Ferns have a broad range of secondary compounds, and although some of these are both unique and well-characterized chemically, this is usually not in the context of an interest in their susceptibility to pathogens. For example, the comprehensive review of acylphloroglucinol compounds in Dryopteridaceae (Socolsky, Hernandez, and Bardon, 2012) describes their anti-helminthic, anti-tumor, molluscicidal, and antibacterial properties but does not mention tests of anti-fungal activity. Other reviews (Vetter, 2018) mention that many secondary compounds have anti-fungal "properties" or "activity" but their role in defense against natural pathogens is rarely considered. Page (2002) discusses the diverse chemical armament of ferns mostly in the context of herbivory, but also notes that ferns survive regimes under which "most flowering plant seedlings would readily 'damp-off' through rapid fungal attack". However, he does not describe experimental work explicitly demonstrating this or suggest a specific mechanism.

Regarding molecular mechanisms, phylogenetic studies have shown that NBS-LRR genes, implemented in the resistance of angiosperms to fungal, oomycete, and bacterial challenge, had their origins prior to the evolution of land plants (Shao *et al.*, 2019). It has also been found that the *Azolla* genome has all the components of the jasmonic and salicylic acid pathways involved in pathogen and herbivore defense in angiosperms (de Vries *et al.*, 2017). A study of a subset of the LRR resistance genes (with the toll interleukin-1 receptor) in a range of plants (Sun *et al.*, 2013), showed that whereas numerous genes were present in dicots, only one copy of one type was present in *Selaginella moellendorffii* Hieron., the only 'fern' for which a sequence was available. Their conclusion that "more information from other species of pteridophytes is needed" was not surprising. There are therefore simply insufficient studies to know if ferns have unique disease resistance mechanisms at the molecular level.

There have also been discussions on why certain phylogenetic lineages may have more pathogen species than others. One hypothesis is that the long-term persistence of pathogens in a lineage is dependent on frequent host shifts among closely related species (Nunn *et al.*, 2004; Thines, 2019). A prediction of this hypothesis is that pathogen species diversity should be higher in lineages that are more diverse, and this was observed by Huang *et al.* (2014) in a study of parasite species richness in carnivores. This should be a very testable hypothesis in ferns given large variation in lineage diversity within the group and evidence for family level variation in pathogen occurrence within the Polypodiales. Another possibility that has been suggested is that older lineages have fewer pathogens because they have had a longer period to evolve effective resistance. Thus, in a study of immune responses and disease incidence in extant species of corals, Pinzon *et al.* (2014) found that species that diverged more than 200 mya had fewer pathogens and stronger constitutive immune functions (measured in a variety of ways) than more recently diverged species. A difficulty with this hypothesis when applied to any one pair of lineages (*e.g.*

ferns vs. angiosperms) is that any two lineages always have the same "age" and therefore would have been exposed to pathogens for identical time periods. However, given that pathogens are often quite lineage specific (Antonovics *et al.,* 2012), a clade retaining ancestral traits may have been exposed to their specialized pathogens longer than a more recently derived clade that acquired new pathogens after divergence. The quite general expectation is therefore that lineages retaining more ancestral traits should have fewer pathogens than derived lineages, and this would be consistent with the present findings.

Unfortunately, these various hypotheses are difficult to distinguish by the single pairwise presented here, but the evidence of family variation in pathogen incidence, even with limited sampling, suggests a broader and more comprehensive analysis of pathogen incidence in ferns in the context of phylogeny may be very fruitful. Other studies have compared the diversity of pathogens on hosts differing in a variety of characteristics: endangered vs. non-endangered (Altizer, Nunn, and Lindefors, 2007), dioecious vs. hermaphrodite (Williams, Antonovics, and Rolff, 2011), rare vs. common: Gibson, Mena-Ali, and Hood, 2010), long vs. short lived (Lindenfors *et al.*, 2007), and invasive vs. non-invasive (Mitchell and Power, 2003). For ferns, this is potentially a rich area for future study, especially as comprehensive databases are being established for trait characteristics in land-plants generally (McGill *et al.*, 2006) and in fungal species (Aguilar-Trigueros *et al.*, 2015). This study has shown that the sporadic nature of the data on pathogen incidence is a major factor making this more difficult, yet this can be overcome by assessment of 'study effort'. I hope the present study provides useful methodological pointers to stimulate future comparative work on ferns and their pathogens at a rigorous quantitative level, as well as encouraging research on fern-pathogen interactions at many levels.

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APPENDIX

1. List of fern species used in the study, and in parentheses the number of citations and number of pathogen species recorded for each. Species names are not italicized, and authority names are as in the USDA plant database (see text).

Adiantum capillus-veneris (791,0); Adiantum caudatum (84,0); Adiantum hispidulum (38,0); Adiantum jordanii (6,1); Adiantum melanoleucum (2,0); Adiantum viridimontanum (7,0); Arachniodes simplicior (6,0); Argyrochosma dealbata (1,0); Argyrochosma incana (3,0); Argyrochosma jonesii (1,0); Argyrochosma limitanea (1,0); Argyrochosma microphylla (1,0); Aspidotis californica (2,0); Aspidotis carlotta-halliae (2,0); Aspidotis densa (5,0); Asplenium abscissum (5,0); Asplenium adiantum-nigrum (163,0); Asplenium adulterinum (35,0); Asplenium cristatum (9,0); Asplenium dalhousiae (27,0); Asplenium heterochroum (6,0); Asplenium monanthes (26,0); Asplenium montanum (35,0); Asplenium pinnatifidum (24,0); Asplenium plenum (3,0); Asplenium resiliens (11,0); Asplenium rhizophyllum (22,0); Asplenium ruta-muraria (824,0); Asplenium scolopendrium (783,0); Asplenium trichomanes (974,0); Asplenium trichomanes-ramosum (10,0); Asplenium tutwilerae (1,0); Asplenium verecundum (1,0); Asplenium vespertinum (2,0); Astrolepis windhamii (2,0); Athyrium americanum (4,1); Athyrium filix-femina (1133,4); Azolla filiculoides (1167,0); Azolla microphylla (251,0); Blechnum occidentale (49,0); Blechnum spicant (175,0); Bommeria hispida (15,0); Botrychium alaskense (4,0); Botrychium boreale (27,0); Botrychium crenulatum (9,0); Botrychium echo (4,0); Botrychium gallicomontanum (9,0); Botrychium jenmanii (4,0); Botrychium lineare (7,0); Botrychium lunaria (442,0); Botrychium matricariifolium (84,0); Botrychium minganense (24,0);

Botrychium mormo (14,0); Botrychium oneidense (13,0); Botrychium pallidum (9,0); Botrychium paradoxum (9,0); Botrychium pedunculosum (4,0); Botrychium pseudopinnatum (3,0); Botrychium pumicola (10,0); Botrychium simplex (69,0); Botrychium tunux (5,0); Botrychium virginianum (145,2); Campyloneurum phyllitidis (15,1); Ceratopteris richardii (337,0); Cheilanthes clevelandii (2,0); Cheilanthes cooperae (3,0); Cheilanthes covillei (1,0); Cheilanthes feei (10,0); Cheilanthes gracillima (7,0); Cheilanthes intertexta (2,0); Cheilanthes lanosa (24,0); Cheilanthes lendigera (3,0); Cheilanthes lindheimeri (6,0); Cheilanthes newberryi (3,0); Cheilanthes notholaenoides (8,0); Cheilanthes pringlei (2,1); Cheilanthes tomentosa (19,0); Cheilanthes villosa (15,0); Cheilanthes viscida (2,0); Cheilanthes wrightii (5,0); Cheiroglossa palmata (8,0); Cryptogramma sitchensis (3,0); Ctenitis submarginalis (9,0); Cyrtomium caryotideum (26,0); Cyrtomium falcatum (127,9); Cystopteris bulbifera (55,2); Cystopteris laurentiana (4,0); Cystopteris protrusa (18,0); Cystopteris tenuis (8,0); Dennstaedtia bipinnata (4,0); Dennstaedtia globulifera (12,0); Deparia petersenii (9,0); Diplazium pycnocarpon (13,0); Drynaria quercifolia (92,0); Dryopteris arguta (17,3); Dryopteris campyloptera (34,0); Dryopteris carthusiana (871,1); Dryopteris celsa (44,0); Dryopteris cinnamomea (25,0); Dryopteris filix-mas (1898,0); Dryopteris fragrans (121,0); Dryopteris intermedia (132,0); Dryopteris rossii (5,0); Gaga arizonica (0,0); Grammitis nimbata (3,0); Gymnocarpium appalachianum (4,0); Gymnocarpium disjunctum (15,0); Gymnocarpium dryopteris (652,1); Gymnocarpium jessoense (13,0); Gymnocarpium robertianum (522,0); Hymenophyllum tayloriae (4,0); Hypolepis repens (14,0); Macrothelypteris torresiana (47,0); Marsilea ancylopoda (8,0); Marsilea minuta (157,0); Marsilea mollis (12,0); Marsilea mutica (11,0); Marsilea oligospora (8,0); Marsilea quadrifolia (237,0); Matteuccia struthiopteris (469,1); Microgramma heterophylla (8,0); Nephrolepis cordifolia (149,0); Nephrolepis exaltata (245,13); Nephrolepis falcata (9,0); Neurodium lanceolatum (2,0); Notholaena aliena (3,0); Notholaena californica (8,0); Notholaena grayi (4,0); Notholaena neglecta (7,0); Odontosoria clavata (1,0); Onoclea sensibilis (353,10); Ophioglossum engelmannii (19,0); Ophioglossum nudicaule (62,0); Ophioglossum pendulum (28,0); Ophioglossum petiolatum (72,0); Ophioglossum polyphyllum (27,0); Ophioglossum vulgatum (603,1); Osmunda claytoniana (79,5); Pecluma dispersa (2,0); Pecluma ptilodon (6,0); Pellaea atropurpurea (36,0); Pellaea breweri (6,0); Pellaea bridgesii (3,0); Pellaea cordifolia (5,0); Pellaea intermedia (6,0); Pellaea lyngholmii (1,0); Pellaea mucronata (9,1); Pellaea ovata (15,0); Pellaea ternifolia (19,0); Pellaea truncata (8,0); Pentagramma triangularis (18,0); Phanerophlebia umbonata (1,0); Phlebodium aureum (56,1); Pityrogramma calomelanos (173,2); Pleopeltis astrolepis (10,0); Pleopeltis polypodioides (22,0); Polypodium calirhiza (4,0); Polypodium glycyrrhiza (59,1); Polypodium hesperium (15,1); Polypodium sibiricum (12,0); Polystichum acrostichoides (106,4); Polystichum braunii (86,0); Polystichum californicum (10,0); Polystichum dudleyi (6,0); Polystichum imbricans (13,0); Polystichum kwakiutlii (1,0); Polystichum scopulinum (5,0); Polystichum setigerum (1,0); Pteridium aquilinum (3214,18); Pteridium caudatum (72,1); Salvinia molesta (620,1);

Salvinia natans (559,0); Salvinia oblongifolia (10,0); Stenochlaena tenuifolia (8,2); Thelypteris augescens (11,0); Thelypteris dentata (53,0); Thelypteris grandis (6,0); Thelypteris hispidula (19,0); Thelypteris nevadensis (6,0); Thelypteris palustris (143,0); Thelypteris patens (12,0); Thelypteris pilosa (13,0); Thelypteris puberula (5,0); Thelypteris quelpaertensis (5,0); Thelypteris reptans (8,0); Thelypteris simulata (10,0); Trichomanes lineolatum (3,0); Vittaria appalachiana (14,0); Vittaria lineata (33,0); Woodsia alpina (62,0); Woodsia appalachiana (3,0); Woodsia glabella (30,2); Woodsia ilvensis (69,0); Woodsia obtusa (36,0); Woodsia oregana (16,1); Woodsia phillipsii (1,0); Woodwardia areolata (15,2); Woodwardia fimbriata (10,1); Woodwardia radicans (58,1); Woodwardia virginica (41,3).

2. List of herbaceous perennial dicots used in the study, and in parentheses the number of citations and number of pathogen species recorded for each. Species names are not italicized, and authority names are as in the USDA plant database (see text).

Abronia ammophila (6,0); Agoseris lackschewitzii (2,0); Alternanthera caracasana (10,0); Ammocodon chenopodioides (3,0); Anaphalis margaritacea (127,8); Anemone drummondii (10,2); Anemone tuberosa (74,1); Angelica breweri (1,3); Antennaria marginata (22,0); Apium nodiflorum (587,0); Apium repens (211,0); Arabis gracilipes (1,0); Arabis hastatula (1,0); Arabis macdonaldiana (2,0); Arabis oregana (3,0); Arabis subpinnatifida (2,0); Arenaria livermorensis (1,0); Arnica amplexicaulis (23,0); Arnoglossum diversifolium (2,0); Artemisia laciniata (82,1); Asclepias eriocarpa (35,0); Asclepias scaposa (1,0); Asclepias subverticillata (12,0); Asclepias syriaca (1295,39); Astragalus americanus (12,0); Astragalus leucolobus (3,0); Astragalus oophorus (2,0); Astragalus phoenix (164,0); Astragalus preussii (11,1); Astragalus ripleyi (0,0); Astragalus soxmaniorum (0,0); Astragalus subvestitus (1,0); Baptisia cinerea (2,0); Bartsia alpina (82,0); Berlandiera subacaulis (3,0); Bidens lemmonii (1,0); Boltonia apalachicolensis (1,0); Boykinia richardsonii (4,0); Callirhoe alcaeoides (2,4); Campanula aparinoides (9,3); Castilleja aquariensis (1,0); Castilleja cinerea (1,0); Castilleja elegans (6,0); Centaurea nigrescens (56,0); Chaptalia tomentosa (9,0); Cirsium clavatum (9,0); Cirsium rusbyi (1,0); Clematis integrifolia (71,1); Conioselinum mexicanum (0,0); Coreopsis grandiflora (151,7); Cymopterus minimus (1,0); Dahlia pinnata (220,20); Dalea searlsiae (8,1); Delphinium alpestre (4,0); Delphinium bakeri (0,0); Delphinium nuttallianum (82,8); Delphinium nuttallii (9,0); Desmanthus bicornutus (11,0); Dianthus carthusianorum (555,0); Draba monoensis (2,0); Draba oreibata (3,0); Dracocephalum thymiflorum (22,0); Drosera linearis (66,0); Encelia nutans (3,0); Enydra fluctuans (44,0); Epilobium luteum (29,1); Erigeron breweri (5,0); Erigeron foliosus (6,0); Eriogonum gracilipes (1,0); Eriogonum panguicense (0,0); Eryngium articulatum (1,0); Eupatorium perfoliatum (140,9); Eupatorium rotundifolium (37,3); Eurybia avita (0,0); Eurybia horrida (1,0); Eurybia merita (2,0); Filipendula vulgaris (346,0); Frasera puberulenta (1,0); Galium humifusum (23,0); Gentiana douglasiana (1,0); Gentiana rubricaulis (0,0); Geranium atropurpureum (4,0); Gratiola hispida (0,0); Grindelia decumbens (1,0); Guardiola platyphylla (3,3); Haplocarpha lyrata

(6,0); Harbouria trachypleura (3,0); Hedeoma oblongifolia (1,0); Hedysarum boreale (38,2); Helianthella microcephala (0,0); Helianthus radula (18,0); Hermbstaedtia odorata (4,0); Heterotheca camporum (6,0); Horkelia sericata (0,0); Hydrastis canadensis (509,5); Hymenoxys lapidicola (1,0); Iliamna grandiflora (1,0); Inula britannica (827,0); Ivesia pityocharis (1,0); Justicia spicigera (47,0); Lasianthaea podocephala (4,0); Lenophyllum texanum (2,0); Leontodon hispidus (674,0); Lesquerella pruinosa (0,0); Lewisia longipetala (5,0); Lewisia nevadensis (3,2); Limonium sinuatum (182,7); Lithospermum mirabile (1,0); Lobelia glandulosa (8,0); Lomatium austiniae (0,0); Lomatium farinosum (4,0); Lomatium juniperinum (1,0); Lotus pedunculatus (1156,0); Ludwigia sphaerocarpa (3,1); Lupinus culbertsonii (2,0); Matelea cynanchoides (0,0); Meehania cordata (6,0); Mentzelia texana (3,0); Mertensia arizonica (8,0); Mimulus lewisii (110,4); Mirabilis glabra (74,0); Mirabilis hirsuta (56,0); Mirabilis oxybaphoides (4,0); Mirabilis texensis (7,0); Monarda russeliana (2,0); Nymphaea tetragona (163,0); Oenothera acutissima (7,0); Oenothera latifolia (30,0); Oenothera nuttallii (9,2); Oxytropis maydelliana (21,1); Packera hyperborealis (0,0); Parnassia cirrata (1,0); Pediomelum pauperitense (2,0); Penstemon alamosensis (0,0); Penstemon albidus (4,2); Penstemon gibbensii (3,0); Penstemon labrosus (1,0); Penstemon pinorum (1,0); Penstemon saxosorum (1,0); Penstemon tenuis (3,0); Penstemon triflorus (5,0); Phacelia arizonica (6,0); Phlox amoena (10,1); Phlox stolonifera (16,0); Physostegia digitalis (5,0); Plantago rugelii (61,17); Polemonium eddyense (1,0); Potentilla drummondii (12,1); Potentilla plattensis (2,0); Potentilla reptans (498,0); Prenanthes nana (4,0); Primula laurentiana (12,0); Pseudognaphalium jaliscense (2,0); Ranunculus allenii (0,0); Ranunculus andersonii (5,0); Ranunculus flammula (764,2); Ranunculus orthorhynchus (2,3); Ranunculus recurvatus (10,6); Rapistrum perenne (12,0); Rorippa coloradensis (0,0); Rudbeckia graminifolia (3,0); Rudbeckia scabrifolia (9,0); Ruellia purshiana (3,0); Rumex pallidus (12,0); Salvia whitehousei (1,0); Satureja hortensis (722,0); Saxifraga hieraciifolia (4,0); Saxifraga tricuspidata (15,1); Schoenocrambe barnebyi (1,0); Scutellaria floridana (5,0); Scutellaria pseudoserrata (2,0); Sedum lanceolatum (31,0); Sedum mexicanum (17,0); Senecio atratus (3,0); Sidalcea covillei (1,0); Silene californica (22,2); Silene rectiramea (0,0); Silene vulgaris (1896,1); Silphium brachiatum (2,0); Silphium glutinosum (1,0); Silphium perfoliatum (299,13); Stachys bigelovii (2,0); Stachys recta (172,0); Stachys stebbinsii (1,0); Stachys sylvatica (144,0); Stellaria irrigua (3,0); Stenandrium dulce (5,0); Symphyotrichum pygmaeum (3,0); Symphytum asperum (119,0); Taenidia integerrima (2,6); Thymophylla micropoides (1,0); Trifolium ambiguum (357,0); Trifolium barnebyi (2,0); Trifolium longipes (22,7); Triosteum angustifolium (6,1); Vernonia blodgettii (2,0); Viola calcicola (5,0); Viola charlestonensis (2,0); Viola umbraticola (1,0); Wyethia helianthoides (1,1).

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On the Spread of Five Nonnative Ferns in Georgia

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Abstract.—Compared to most other groups of vascular plants, ferns have been neglected with respect to the potential for nonnative species to invade native plant communities and displace native species. Targeted collecting in the Piedmont of northeast Georgia uncovered five nonnative ferns that represent range extensions or provide clear confirmation of naturalization. Examination of herbarium specimens from Georgia revealed that some earlier records, as well as some overlooked records, were based on misidentifications. The species involved (*Cyrtomium fortunei*, *Deparia petersenii*, *Dryopteris erythrosora*, *Macrothelypteris torresiana*, and *Pteris multifida*) are all introductions from temperate regions in East Asia and represent escapes from cultivation. Study of Georgia collections held in herbaria from throughout the Southeast allowed determination of the first appearance and subsequent range expansion of the five species over time. These data and information about mating systems, ploidy level, and ecological requirements of each species allowed inferences to be made about their probable spread in the future, including effects of climate change.

Key Words.—*Cyrtomium fortunei*, *Deparia petersenii*, *Dryopteris erythrosora*, *Macrothelypteris torresiana*, *Pteris multifida*

Despite growing interest in research on invasive nonnative plants, relatively few studies have focused on ferns. As a case study, therefore, I have chosen to document the spread of five nonnative fern species in Georgia (*viz.*, *Cyrtomium fortunei* J. Sm., *Deparia petersenii* (Kunze) M. Kato, *Dryopteris erythrosora* (D.C. Eaton) Kunze, *Macrothelypteris torresiana* (Gaudich.) Ching, and *Pteris multifida* Poir.). None of these appear to pose as large a threat, however, as *Lygodium japonicum* (Thunb.) Sw., a climbing fern that can grow over other vegetation and shade it out, as well as laddering fire into tree canopies (Wyatt and Wyatt, 2013). This species is the only fern listed as a Category 1 invasive species by the Georgia Exotic Pest Plant Council (Gray *et al.*, 2010), signifying that it poses a serious problem in natural areas by extensively invading native plant communities and displacing native species. The only other ferns listed are two aquatic species, *Salvinia molesta* D.S. Mitchell and *Marsilea minuta* L., which are not yet deemed to be a serious problem.

Compared to many other states in the Southeast, Georgia has had significant attention paid to its fern and lycophyte flora, beginning with McVaugh and Pyron's (1951) *Ferns of Georgia* and, after a gap of 35 years, Snyder and Bruce's (1986) *Field Guide to the Ferns and Other Pteridophytes of Georgia*. Of the five species considered in this study, only *P. multifida* was included in McVaugh and Pyron's (1951) book. Snyder and Bruce (1986) added *C. fortunei*, *D. petersenii* (as *Diplazium japonicum* (Thunb.) Bedd.), and *M. torresiana* (as

Thelypteris torresiana (Gaudich. in Freyc.) Alston). *Dryopteris erythrosora* is a very recent addition to the flora of Georgia, based partly on my collections (Zomlefer *et al.*, 2018). It seems appropriate, after another interval of 34 years, to evaluate the extent to which these ferns have expanded their ranges and moved into new geographical areas and ecological settings as documented by new collections from across the state held in various herbaria in the Southeast.

Materials and Methods

Over the course of several years I sampled populations of these five ferns from numerous sites in Clarke and Oconee counties in northeast Georgia with a view to documenting their occurrence in the Piedmont. Specimens were pressed and dried and deposited in the University of Georgia Herbarium (GA). Sites were targeted based on previous knowledge of habitats in which each species might be expected to occur.

To get a broader view of the range of these species in Georgia, I examined all of the imaged specimens of the five fern species (including relevant synonyms) represented in herbaria included in SERNEC (Southeastern Regional Network of Expertise and Collections: sernecportal.org/portal/index.php). Relevant data were recorded for each specimen, including date of collection, county, collector, and habitat. Although Georgia is the largest state in the Southeast, it is finely divided into 159 counties of varying size. From this information I could ascertain when and where each species first appeared in the state and the rate and pattern of its spread over time. I also examined all of the specimens of these species at GA, correcting a few misidentifications and noting carefully all of the label information pertaining to habitat. These data were used to test several hypotheses regarding the likelihood of further range expansion based on the ecology and mating systems of the five species. Specifically, species that are polyploid, apogamous, and not restricted to particular substrates are predicted to be more likely to colonize new counties and pose problems in the future.

Results

My sampling of local populations confirmed the presence of all five species in the Piedmont of northeast Georgia. Some of these represented significant range extensions (*e.g.*, 170 km to the north for *Deparia petersenii*) or first collections from outside the Coastal Plain (*e.g*, *D. petersenii*). Others confirmed the species to still be present after an extended period of time (*e.g.*, *Pteris multifida* more than 40 years since the most recent collection in Clarke County).

Tabulation of the results from searching SERNEC turned up 166 collections of *Macrothelypteris torresiana* from Georgia, of which 72 were unique, from a total of 42 counties. There were no collections made prior to 1951, 37 in the period from 1951-1986, and 35 from 1986-present. Breaking this time frame into decades yields the following: 1950s, 1; 1960s, 12; 1970s, 7; 1980s, 28; 1990s, 5; 2000s, 6; and 2010s, 13. The species appears first in Coastal Plain

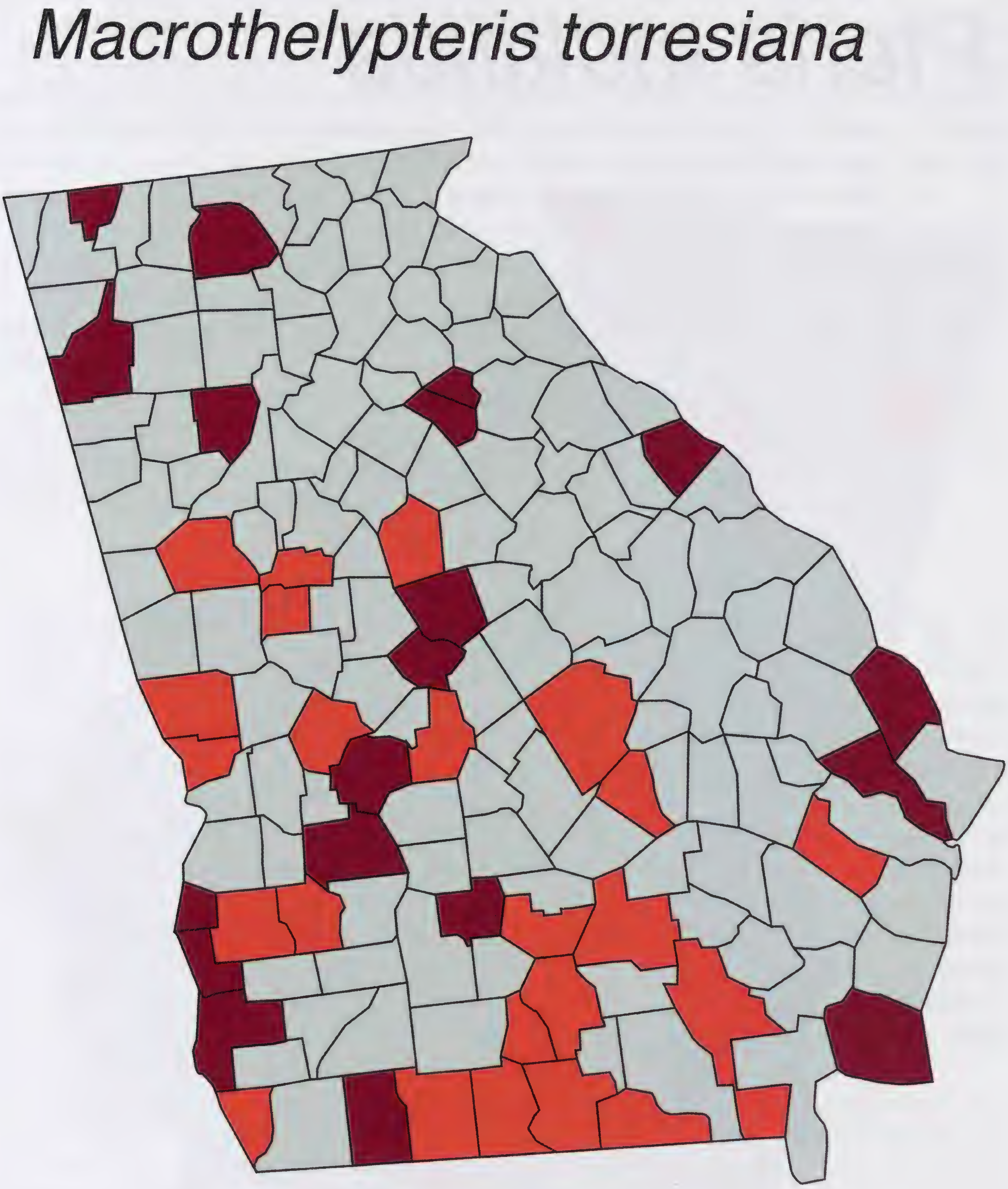

FIG. 1. Distribution of *Macrothelypteris torresiana* in Georgia. Counties colored orange represent herbarium records dating to 1951-1986, and counties colored dark red, 1986-present.

counties adjacent to Florida, later spreading to the Piedmont, and finally to the Ridge and Valley province (Fig. 1).

For *Pteris multifida*, searching of the collections on SERNEC yielded 48 specimens, of which 29 were unique. These represented 11 different counties, with one county, Chatham, being represented by 13 separate collections. The first collection was made in 1893 in Richmond County. Prior to 1951 five collections were made from three counties. From 1951 to 1986, 18 collections

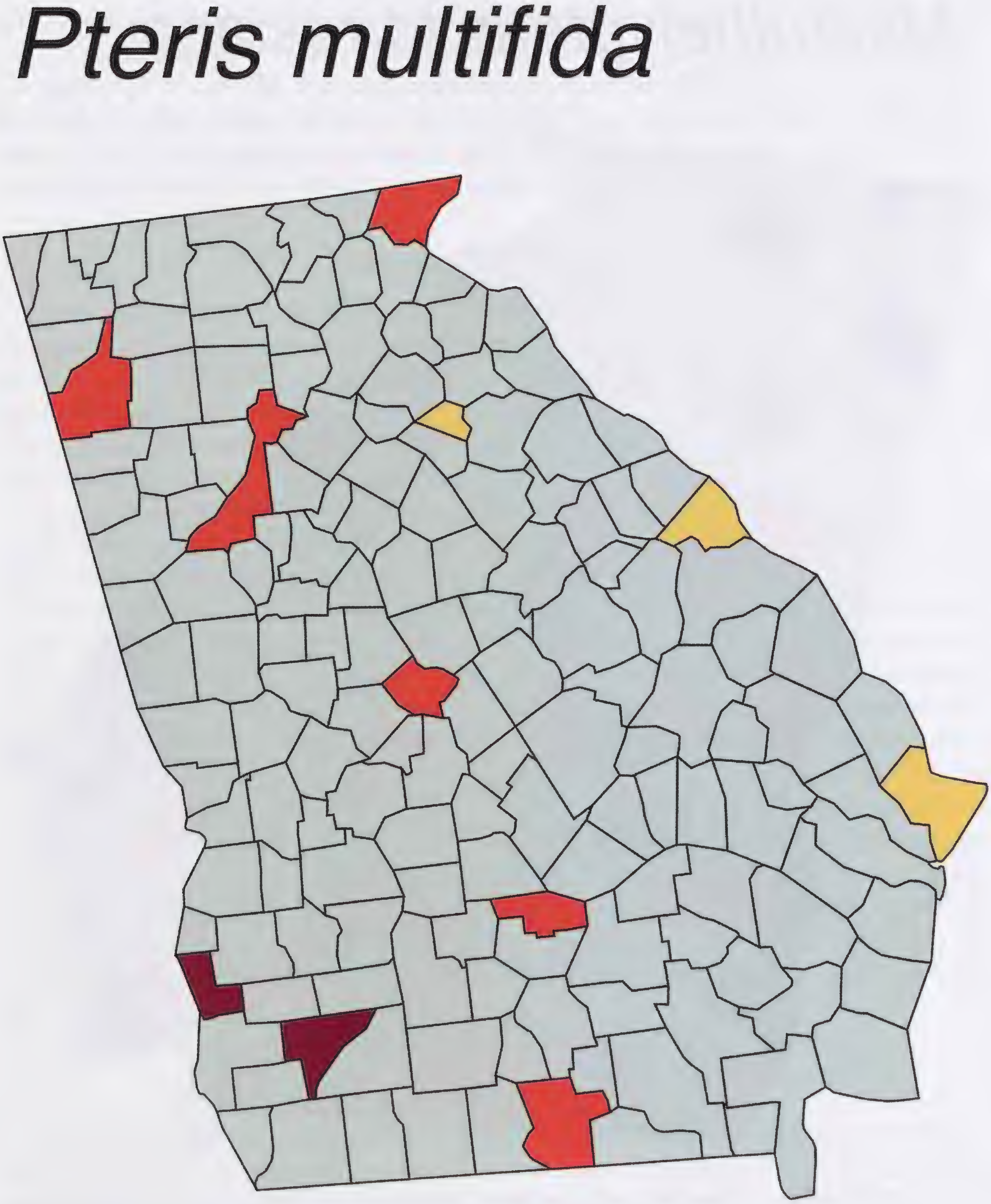

FIG. 2. Distribution of *Pteris multifida* in Georgia. Counties colored yellow represent herbarium records prior to 1951. Counties colored orange represent herbarium records dating to 1951-1986, and counties colored dark red, 1986-present.

were added representing six new counties. And from 1986 to the present, six new collections were made adding two new counties (Fig. 2). Spread of this species has occurred from east to west in Georgia. The accumulation of collections by decades is as follows: 1950s, 4; 1960s, 4; 1970s, 7; 1980s, 2; 1990s, 1; 2000s, 1; and 2010s, 1.

Deparia petersenii was represented by 19 collections in SERNEC from a total of 10 counties. The first collection was made in 1980, with five more being added prior to 1986. From 1986 to the present, 13 new collections were added representing four new counties. By decades the breakdown is: 1980s, 9; 1990s, 0; 2000s, 7; and 2010s, 3. Until 2016, when I collected this plant from the Piedmont, all collections had come from Coastal Plain counties (Fig. 3).

Searching of SERNEC yielded only nine collections of *Cyrtomium fortunei*, the earliest having been made in 1958 in Floyd County. From then to 1986 five collections were added, all from Clarke County. Since 1986 four new collections have been made, one from Floyd County and the rest from Clarke (Fig. 4).

Dryopteris erythrosora is represented in SERNEC by 19 total collections, of which seven are unique, and all have been made since 2010. Moreover, all collections to date have come from five counties in a small area of the north-central Piedmont (Fig. 5).

Discussion

Macrothelypteris torresiana.—This species was first introduced to the United States in 1904, having escaped from cultivation in Florida (Leonard, 1972; Mickel, 2003), from which it spread to Alabama, Arkansas, Georgia, Louisiana, Mississippi, South Carolina, and Texas (Diggs and Lipscomb, 2014). More recently it has been reported from Tennessee (Beck and Van Horn, 2007; McCoy, 2008), Kentucky (Gorman, Bruton, and Estes, 2011), and Virginia (Wieboldt *et al.*, 2011). McVaugh and Pyron (1951) did not list *M. torresiana* as present in Georgia. Oliver (1955), however, reported a population from Nassau County, Florida, growing less than 300 m from the state line and predicted that it would soon be found in Georgia. Duncan and Blake (1965) subsequently discovered this species growing abundantly in natural habitats in three counties in Georgia, concluding that it "definitely is a part of our natural flora." By the time Snyder and Bruce (1986) updated the fern flora, plants were known from 23 counties, an extremely rapid expansion of the range.

In the 34 years since publication of Snyder and Bruce's (1986) book, 19 additional counties have been added. These localities have extended the range of this species into the Piedmont and Ridge and Valley physiographic provinces. Most early collections came from the Coastal Plain, consistent with the pattern seen in *Lygodium japonicum* (Wyatt and Wyatt, 2013). It is questionable how much farther north these plants can grow, as Mickel (2003) noted that they are only recommended for USDA hardiness zones 8 to 10. Of course, it is possible that their expansion northward will be aided by global warming, and the region in which I found these plants has recently been reassigned to zone 8 after many decades of being assigned to zone 7b. Moreover, for some plants it has been suggested that climate preferences can alter during range shifts (Atwater, Ervine, and Barney, 2018).

The chronology of collections of *M. torresiana* is informative regarding the rate of spread of this species in the state. Prior to 1960, only one collection had

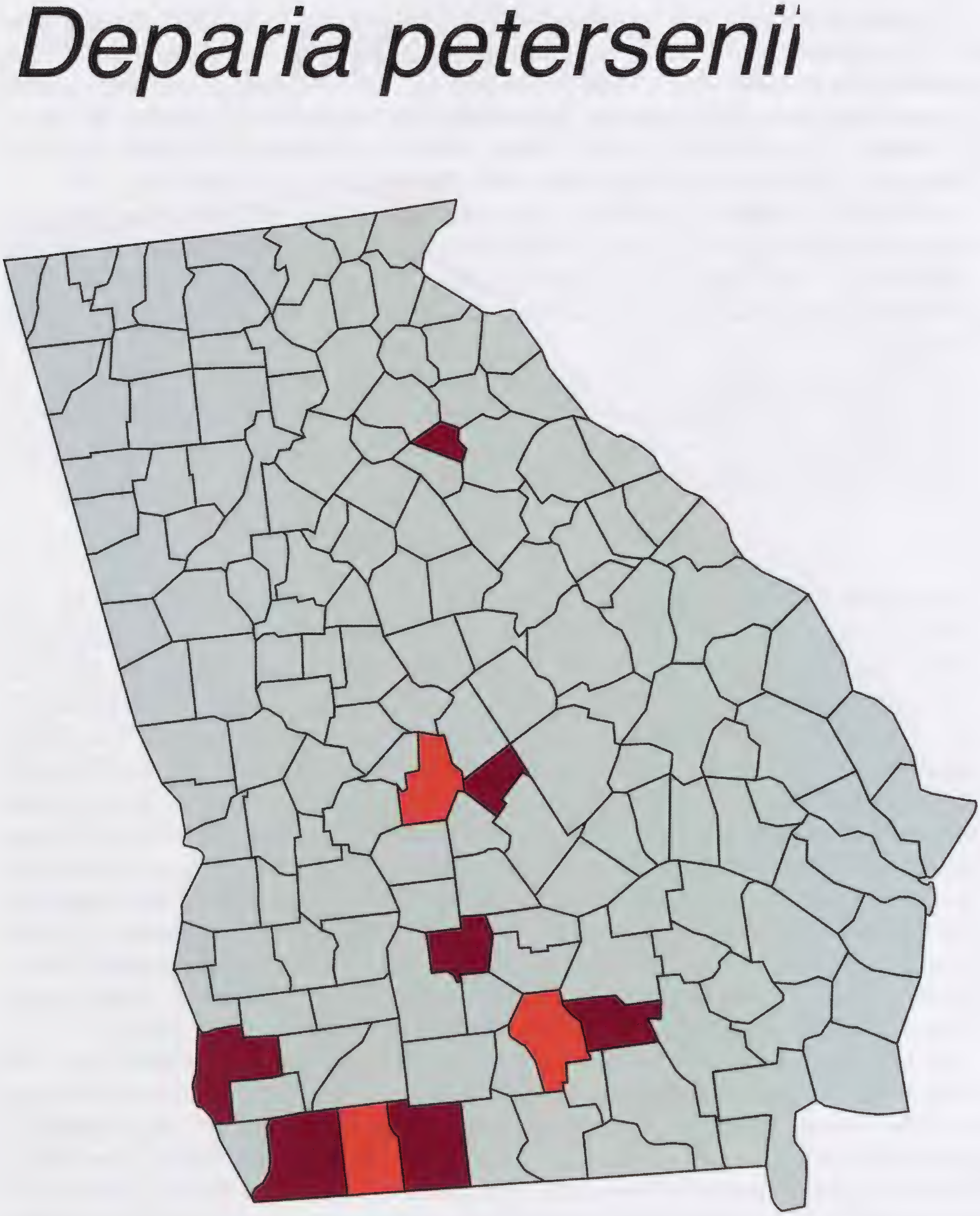

FIG. 3. Distribution of *Deparia petersenii* in Georgia. Counties colored orange represent herbarium records dating to 1980-1986, and counties colored dark red, 1986-present.

been made. The pace picked up somewhat in the 1960s and 1970s when this species was discovered in nine additional counties based on 19 collections. In the 1980s, 28 collections were added representing 20 new counties, but only seven more were added in the 1990s. Since 2000 only five new counties have been recorded based on 19 collections. Some of this pattern may be explained

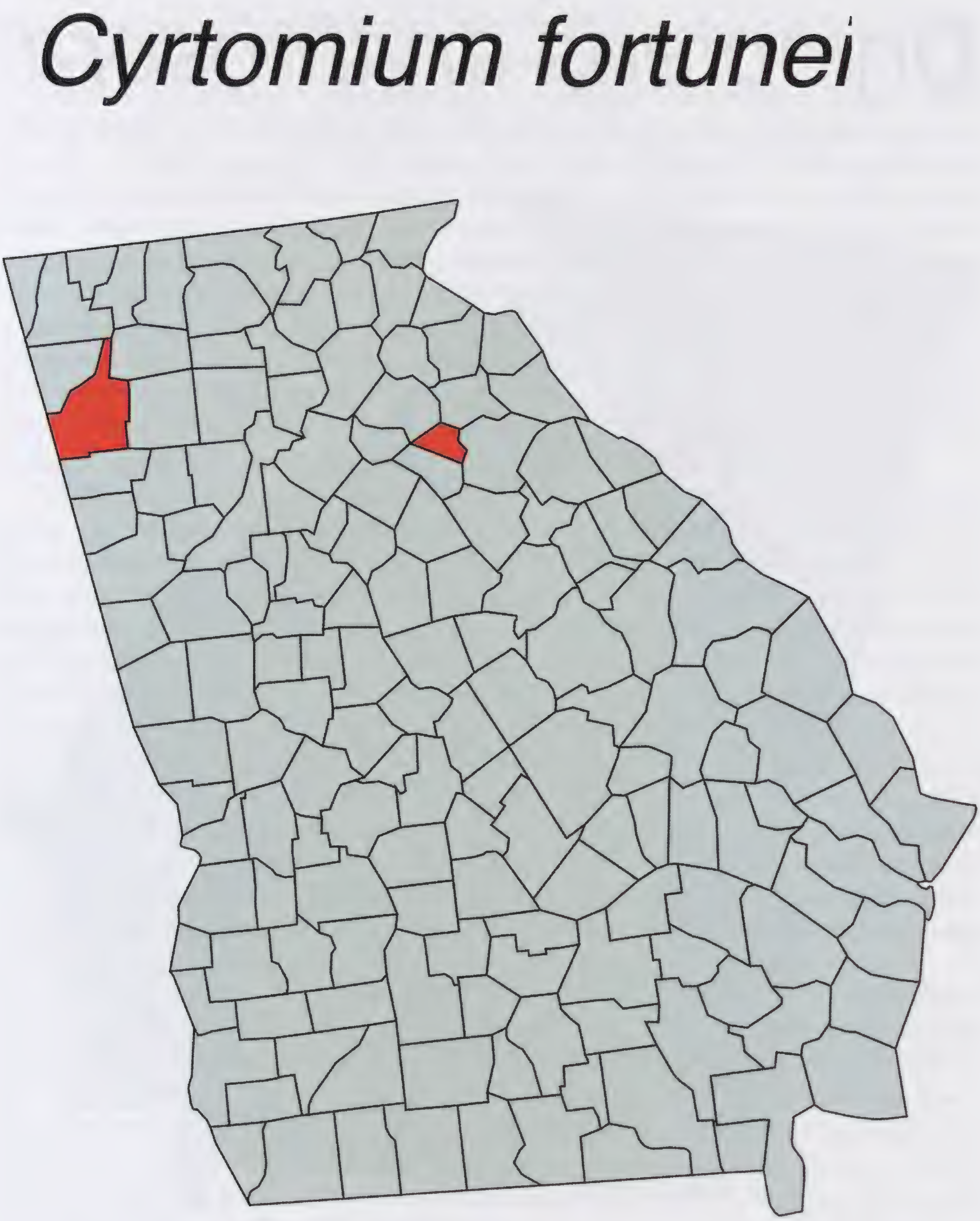

FIG. 4. Distribution of *Cyrtomium fortunei* in Georgia. Counties colored orange represent herbarium records dating from 1951-present.

by the intensity of collecting, which apparently peaked in the 1960s, 1970s, and 1980s, declining steeply thereafter. In South Carolina, though plants have been known to occur in the vicinity of Charleston for many years (Leonard, 1972; Smith, 1993), it was not until Horn's (2006) report that the species was recorded in the Piedmont.

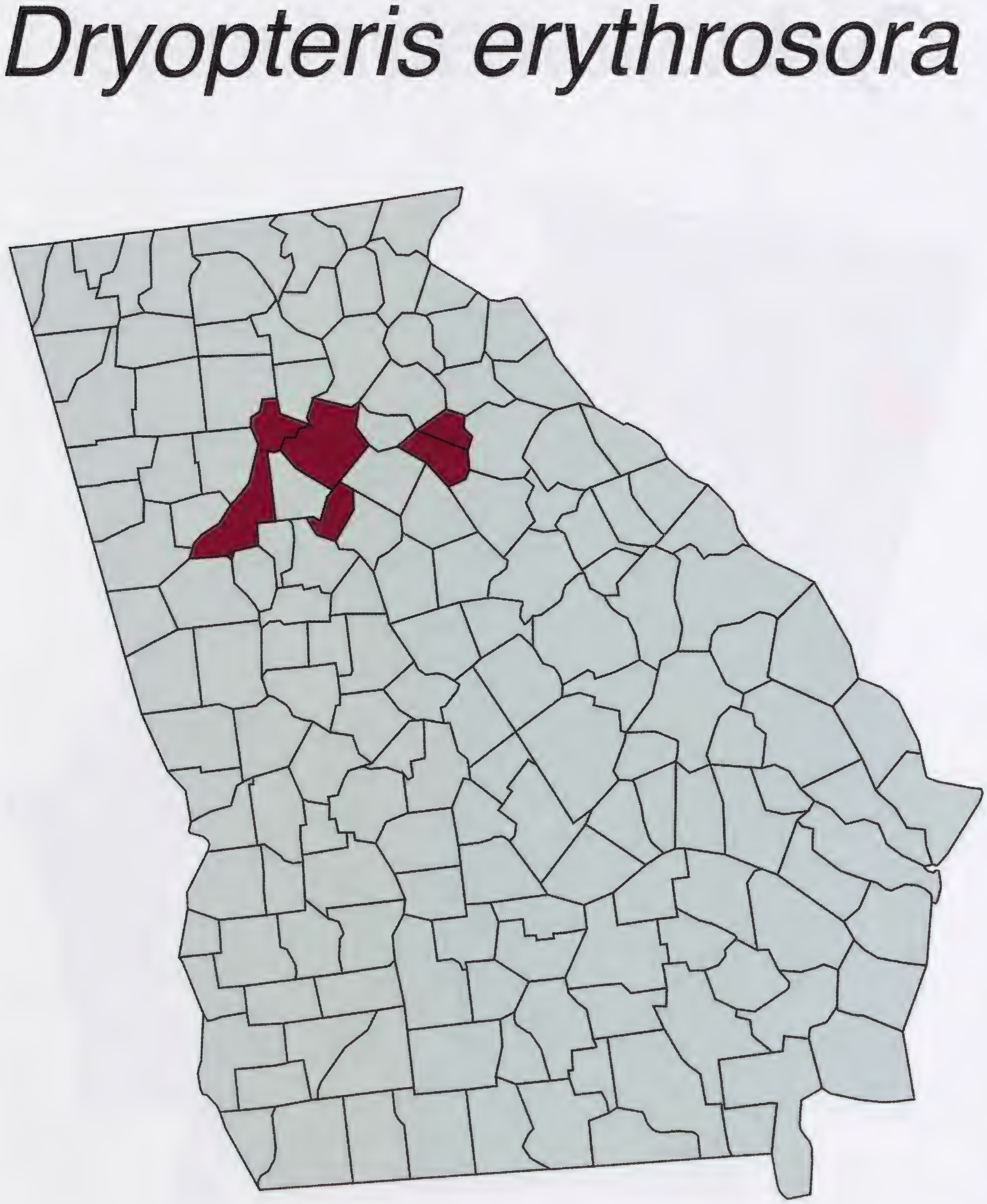

FIG. 5. Distribution of *Dryopteris erythrosora* in Georgia. Counties colored dark red represent herbarium records dating from 2011-present.

Hoshizaki and Moran (2001) note that *M. torresiana* is native to Asia and Africa, mostly in tropical to subtropical climates (Smith, 1993). It occupies similar environments in southern Mexico (Aguilar-Dorantes *et al.*, 2015), Central America, and South America as far as southern Brazil and northern Argentina (Rosetto and Vieira, 2013). It also occurs in the West Indies (Gaddy,

2013) and has been reported from New Zealand as a possible case of long-distance dispersal (de Lange and Crowcroft, 1997).

Voucher Specimens Collected. U.S.A. Georgia: Oconee Co., Watkinsville Town Cemetery on Simonton Bridge Road, marshy ground along small stream just N of the cemetery, 1 Jul 2014, *Wyatt 1884*. Beneath bridge on Macon Highway over McNutt Creek, sandy soil in alluvial plain of the creek, 21 Jul 2014, *Wyatt 1893*. In ditch on south side of Harden Hill Road about 0.2 km W of its intersection with US 441 in Watkinsville, near a small creek, 16 Sep 2016, *Wyatt 1900*. Clarke Co., on White Trail along Oconee River about 100 m upstream from power line cut, State Botanical Garden of Georgia, in sandy alluvium close to trail, 20 Oct 2016, *Wyatt 1901*. Harris residence at 140 Skyline Way off Timothy Road in Athens, abundant along a small creek, 5 Nov 2016, *Wyatt 1902*. Wooded ravine off Hanover Circle in Hanover Estates in Athens, scattered plants on creek bank, 12 Nov 2016, *Wyatt 1905*.

Pteris multifida.—This species is a weedy, colonizing tetraploid originally native to eastern Asia (Murakami *et al.*, 1997). In the United States it is naturalized from Maryland south to Florida and west to Arkansas and Texas, but also occurs in Illinois, Indiana, Kentucky, and New York (Diggs and Lipscomb, 2014). Riefner and Smith (2016) have more recently documented the species growing outside of cultivation and naturalized in southern California. It is also known from the West Indies, Argentina, and Brazil (Nauman, 1993). Celesti-Grapow *et al.* (2009) listed it as a “neophyte” in Italy. The first report of *P. multifida* in the United States was in 1868 from a Huguenot cemetery in Charleston, South Carolina (Nelson, 2000). Because this plant was cultivated on many plantations in the Deep South, it sometimes escaped not just to rock walls and other masonry structures but into wooded areas where it became naturalized (Correll, 1956). There are many cultivars, some of which are also known to have established in natural areas (Riefner and Smith, 2016).

Pteris multifida was included in McVaugh and Pyron’s (1951) list of the ferns of Georgia, their map showing it in five widely scattered counties. According to Diddell (1953), there were numerous dense colonies of this fern growing in the old Colonial Cemetery in Savannah that apparently “had been growing there for a good many years.” Snyder and Bruce (1986) added only three additional counties in the 35 years after McVaugh and Pyron’s (1951) compilation. My examination of specimens at GA added only Floyd County, notably an extension into the northwestern corner of the state, to this list. Like most collections from Georgia, this one was made from mortar and brick (on a church in Rome). Since 1986 two more counties, Clay and Baker, in the southwestern corner of the state have been added to the list. This fern does not seem to be spreading aggressively in the state and appears to be largely restricted to human constructions, occasionally spreading to circumneutral soils as noted by Lellinger (1985).

Voucher Specimen Collected. U.S.A. Georgia: Oconee Co., beneath bridge on Macon Highway over McNutt Creek, in crevices of rock wall, representing the remains of former textile mill dam, 28 Apr 2014, *Wyatt 1891*; 21 July 2014, *Wyatt 1892*.

Deparia petersenii.—It is unclear when this fern was first introduced to North America, but it appears to be a recent introduction and so far the plants have not spread very widely. It is native to southeastern Asia, but it has spread widely in the Old World in tropical and subtropical areas and is adventive in Australia (Shinohara *et al.*, 2006). Shinohara, Takamiya, and Murakami (2003) showed that "two biological species are contained in Japanese *D. petersenii*," and subsequent studies have revealed that there are four cytotypes (diploid, tetraploid, pentaploid, and hexaploid). The widespread tetraploid has arisen independently at least twice (Shinohara *et al.*, 2006).

In North America this fern is known only from a small region in the Coastal Plain of southwestern Georgia, extreme northern Florida, and the southeastern corner of Alabama (Kato, 1993). Presumably, all of these records are of plants that persisted or escaped from cultivation. This species was not listed by McVaugh and Pyron (1951). Snyder and Bruce's (1986) map showed it (as *Diplazium japonicum*) in only three counties, all in the Coastal Plain. My update based on specimens in SERNEC shows that this species, 34 years later, has spread to six additional counties adjacent to those where it was found previously. In addition, my own collecting has added another county (Clarke), extending the range more than 170 km into the Piedmont of northeast Georgia.

There is disagreement about the extent to which *D. petersenii* has become naturalized in the United States. Morton and Godfrey (1958) stated that the species is naturalized in Florida, but Long and Lakela (1976) questioned whether it is truly naturalized there. In Georgia the first collection made (in 1980) was from a "dried creek bed under a bridge," obviously a disturbed site. The second collection, not long after, was "along a stream in dense woods," and most subsequent collections have come from areas that are not heavily impacted by humans. In Hawaii *D. petersenii* has been found invading native low-elevation tropical montane wet forests on Mauna Kea (Tweiten *et al.*, 2014). It also colonized a constructed wetland (White, Taylor, and Damrel, 2012) in Grady County, Georgia, one of the first counties from which the species was reported (in 1981).

Voucher Specimen Collected. U.S.A. Georgia. Clarke Co., Harris residence at 140 Skyline Way off Timothy Road in Athens, numerous scattered clumps of plants along creek especially on rocky banks, 5 November 2016, *Wyatt 1903*.

Cyrtomium fortunei.—This fern was introduced early to North America. Most accounts cite Christensen's (1930) date of 1866, but Brown (2008) claims that plants were imported to California as early as 1858. All authors do agree, however, that Robert Fortune first collected the species in southeast China in 1845. It was introduced as an ornamental to Japan before being sent to London and described by John Smith in 1866. In the United States *C. fortunei* was very popular in the horticultural trade, with flats of 200 plants selling for $2 per flat (Dreer, 1914). Landry *et al.* (1979) noted that, more recently, "it has been superseded by more attractive species such as *C. falcatum*." Diggs and

Lipscomb (2014) state that it is native to Japan, Korea, southeast China, Vietnam, and eastern Thailand.

In the United States *C. fortunei* is known from Georgia, Louisiana, Mississippi, Oregon, and South Carolina (Yatskievych, 1993). It has also been discovered more recently in Alabama (Barger *et al.*, 2012), Arkansas (Peck, 2011), and Kentucky (Hulsey and Meier, 2011). Hulsey and Meier (2011) also state that it has been observed in Tennessee by Dwayne Estes (personal communication). It certainly appears that this fern is capable of growing in colder climates than it experiences in its native range, though Hoshizaki and Moran (2001) listed it as only semi-hardy in USDA hardiness zones 7 and 8. There is also disagreement regarding the extent to which *C. fortunei* is truly naturalized in these areas. For example, Landry *et al.* (1979) criticized earlier reports such as MacDougal's (1976) observation of 70 plants "growing in association with *C. falcatum* on a moist north-facing brick wall in a cemetery in downtown Charleston." They stated, "in our opinion these plants qualify as escapes or adventives, but are scarcely naturalized." In contrast, Lellinger (1985) and Diggs and Lipscomb (2014) clearly accept the species as "naturalized" in the United States.

Elsewhere there are similar debates about the hardiness, naturalization, and invasiveness of *C. fortunei*. The species was first reported in the Netherlands in 1992 and in Belgium in 2002, but by 2008 nine sites in the Netherlands and 19 in Belgium were counted by Denters and Verloove (2008). They noted that this rapid spread was limited to "urban habitats where frostbite is less likely." Comin *et al.* (2011) attempted to assess invasiveness of nonnative plants, including *C. fortunei*, using a diachronic approach that looked at the rate of new site colonization over time in northeast Italy. The scores attributed to this fern, which was first observed in 1979, placed it in Group III with other plants classified as "non-invasive." Gholipur and Greuter (2010) discovered a stand of *C. fortunei* on sandstone rocks in a natural area on the northern slope of the Elburs range in northern Iran and suggested that the species is indigenous, despite the extreme geographical disjunction. They rejected the possibility of long-distance dispersal, partly because the species is not known to be cultivated in Iran.

Cyrtomium fortunei was not listed for Georgia by McVaugh and Pyron (1951). Snyder and Bruce (1986) reported it in three counties (Chatham, Clarke, and Colquitt), but this range was based on misidentifications. Only the specimen from Clarke was correctly identified; the others proved to be *C. falcatum* (L.f.) C. Presl. My checking of other specimens in GA and VSC also turned up three specimens from Floyd County, identified as *C. falcatum*, that were actually *C. fortunei*. Two came from the Darlington School campus in Rome and were collected in 1958 from brick walls, with numbers of plants ranging from one to six. The other specimen was from a rock wall in Rome and was collected in 2005. The three collections from Clarke County were made in 1958, 1963, and 1964 and came from rock foundations of railroad embankments on the campus of the University of Georgia.

My own collections were also from rock walls, one in a cemetery and one from a decaying textile mill dam. Both were thriving colonies of many plants, including a population at the dam of thousands that must have been founded many, perhaps one hundred or more, years ago. The site is where the oldest of Athens's textile mills, originally called the Georgia Factory, was organized in 1830 by John White (Rice, 2001). In 1929, however, the newly named Whitehall Mills went bankrupt, and the dam and associated structures fell into disrepair. There is no doubt that this species has become naturalized in this area.

Voucher Specimens Collected. U.S.A. Georgia. Clarke Co., Oconee Hill Cemetery off East Campus Road in Athens, in crevices of rock retaining wall about 75 m NW of West Hill Lot 181 grave of Wilson Lumpkin above the North Oconee River, 11 Mar 2014, *Wyatt 1880.* White Dam on E side of the North Oconee River about 2 km SSE of Whitehall Road, alluvial forest with river birch, sycamore, box elder, winged elm, and Carolina silverbell, in crevices of rock walls of dam, 13 Mar 2014, *Wyatt 1882.* White Dam on W side of the North Oconee River just below Devil's Ford Road in the Whitehall Forest, alluvial forest with river birch, sycamore, box elder, winged elm, and Carolina silverbell, abundant on rock walls of old mill race spreading to alluvial soil, 20 Mar 2014, *Wyatt 1885.*

Dryopteris erythrosora.—This fern is very popular in the horticultural trade and is "hardy with minimal protection in all but the coldest of the United States" (Lellinger, 1985). Mickel (2003) states that it can grow in USDA hardiness zones 5 to 8. It is a recent introduction to the United States, prompting Olsen (2007) to remark "40 years ago, when amazingly by today's market standards, it was unavailable." Olsen (2007) gives its native range as East Asia from Japan, China, and Korea to the Philippines.

The first instance of *D. erythrosora* escaping from cultivation was reported by Simpson, Crank, and Peck (2008). They found a single plant at the edge of an old timber access road in Hot Springs County, Arkansas. They suggested that it must have colonized via long-distance dispersal of spores because the nearest potential source of cultivated ferns was more than 16 km to the east. More recently, Rothfels *et al.* (2012) found two plants of *D. erythrosora* growing in a disturbed suburban lot in Durham County, North Carolina. They noted that there was no "indication of it (or anything else) having been planted on the site or in the vicinity." In Georgia neither McVaugh and Pyron (1951) nor Snyder and Bruce (1986) listed this fern as occurring in Georgia. Zomlefer *et al.* (2018) reported it, as an escape from cultivation, from six sites in three counties based on specimens collected by James R. Allison and myself. All of these were very recent collections dating from 2012 to 2017 and came from suburban landscapes mostly associated with stream banks. Since their report of plants from Clarke, Gwinnett, and Rockdale Counties, I have added Oconee County to the list.

Voucher Specimens Collected. U.S.A. Georgia. Clarke County. Tanyard Branch just W of Lumpkin Street on the University of Georgia campus, in soil on banks of stream just above the rock-lined channel, 11 Mar 2014, *Wyatt 1881.*

Oconee County. West bank of McNutt's Creek at old bridge connecting Tanglebrook Drive and Mal Bay Road, in shallow sandy soil at end of concrete abutment above creek level, 5 Nov 2016, *Wyatt 1904*.

Conclusions

It is worth noting that Clarke County is the smallest of 159 counties in Georgia with a land area of 309 km^2. Oconee County, which is adjacent to Clarke, ranks 145th in size with a land area of 376 km^2. Moreover, this part of the state, being close to the University of Georgia, is certainly one of the best-collected areas in the state. It served as home base for McVaugh and Pyron (1951) and for Snyder and Bruce (1986), who produced book-length treatments of the fern and lycophyte flora. And the most prolific collector of Georgia plants, Wilbur Duncan, spent his long career at the University of Georgia, often focusing on ferns (*e.g.*, Duncan, 1955; Duncan and Blake, 1965). It is therefore remarkable that my recent collecting in this small, well-collected area uncovered a number of new records and range extensions. It seems likely that these species also occur in many additional localities between Clarke and Oconee counties and previously known sites. It is also likely, since all five of the species of interest are nonnatives, that they simply were not present in the northeast Piedmont of Georgia until recently, *Cyrtomium fortunei* and *Pteris multifida* being the exceptions. Another species that fits this pattern is *Lygodium japonicum*, which Wyatt and Wyatt (2013) reported from Oconee County.

All five of the nonnative species under consideration are originally native to temperate regions of East Asia, and all have been introduced to North America as escapes from cultivation. Their spread is facilitated by the potential for long-distance dispersal of their spores, which can travel over great distances (e.g., Ranker, Floyd, and Trapp, 1994; Perrie and Brownsey, 2007). The likelihood of range expansion is enhanced for species that are apogamous, like triploid *C. fortunei* (2n = 123: Yatskievych, 1993) and tetraploid *P. multifida* (2n = 116: Nauman, 1993). This may also be true for *Dryopteris erythrosora*, which Rothfels, Sigel, and Windham (2012) assumed to be an apogamous triploid. It is also possible, however, that cultivated North American plants of *D. erythrosora* are apogamous tetraploids (2n = 164), as such plants have recently been reported from Japan by Hori and Murakami (2019). *Macrothelypteris torresiana* (2n = 124: Vijaykanth *et al.*, 2018) is a sexual tetraploid, which Mishra *et al.* (2019) argued might diminish its "capacity for colonization." *Deparia petersenii* (2n = 160: Shinohara *et al.*, 2006) is also likely to be a sexual tetraploid, but is known to exist as diploid, tetraploid, pentaploid, and hexaploid cytotypes. Kuo *et al.* (2016) noted that polyploid species of *Deparia* "are potentially better dispersers than their sexual diploid relatives," but the chromosome number of North American plants has not been determined.

Of the five species, *C. fortunei* and *P. multifida* have been grown in the state for the longest period of time, having been introduced in the mid-nineteenth century. They seem to have been slow to spread, but are certainly naturalized and form large populations in certain areas. Given that they are specialists on rock or brick walls and require rich soil with a higher pH than is commonly encountered in Georgia, they seem to be restricted primarily to human-altered habitats. They do not appear to be highly invasive or likely to exclude native species. This conclusion is consistent with the finding of Comin *et al.* (2011) that *C. fortunei* is not invasive in northern Italy. Given that this species has fallen out of favor in the horticultural trade, which is pushing instead its close relative, *C. falcatum*, it seems unlikely to spread much more in the future. Celesti-Grapow *et al.* (2009) also concluded that *Pteris multifida* in Italy is not invasive, classifying it as "casual" (*i.e.*, an alien plant that must rely on repeated introductions to persist). That these two species are less invasive than some others is counter to predictions in that both are apogamous.

The other apogamous species, *D. erythrosora*, is probably too recently introduced to allow an informed prediction of how widely it might spread in the future. Two factors suggest it might have tremendous potential. First, it is wildly popular as an ornamental planting and undoubtedly hundreds of thousands, if not millions, of plants will be installed in landscapes all over Georgia and the Southeast. Second, this species is hardy over most of the United States. Similarly, it is likely too soon to tell how widely *D. petersenii* will spread, but the fact that it was unknown in Georgia prior to 1980 and is now known from ten counties suggests that it could be highly invasive. Finding it in northeast Georgia, well into the Piedmont, indicates that it can tolerate colder climates. Moreover, it grows on the banks of undisturbed streams in intact native forest. This is also the case in Hawaii where it is reported to invade low-elevation native forest of *Acacia koa* A. Gray and *Metrosideros polymorpha* Gaudich. (Tweiten *et al.*, 2014).

Comin *et al.* (2011) proposed that colonization can be looked at as a sigmoid curve with three phases: a lag phase of initial establishment with low spread; an expansion phase marked by increasing rates of spread; and a saturation phase when rates of spread reach a plateau. Of the three species considered in this study that seem to have potential to spread, *D. erythrosora* would seem to be in the lag phase and *D. petersenii* would seem to be moving from the lag phase into the expansion phase. On the other hand, *M. torresiana* clearly appears to be in the expansion phase, possibly beginning to enter the saturation phase. This species arrived from Florida in the 1950s, spread to nine more counties in the 1960s and 1970s, then exploded in the 1980s and 1990s to occupy 27 additional counties. Since then, however, it has only spread to five more counties. It is possible, however, that this pattern is an artifact of a decline in collecting intensity following the 1980s. Another factor to be considered is climate change. A shift to warmer temperatures would make it possible for many ferns that are presently restricted to warmer regions of the Southeast, like *M. torresiana*, to spread northward.

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Phylogenetics of Hawaiian *Diplazium* (Athyriaceae: Polypodiales)

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Abstract.—Hawaii is home to four species in the fern genus *Diplazium*: *Diplazium arnottii, D. esculentum, D. molokaiense*, and *D. sandwichianum*. Three are endemic to the Hawaiian Islands, and one is introduced and naturalized (*D. esculentum*). They vary in frequency, from very abundant (*D. sandwichianum*) to rare and critically endangered (*D. molokaiense*). Prior to this work, the phylogenetic relationships of only *D. esculentum* had been estimated due to lack of information available about these species. In this study, we inferred phylogenetic relationships of the Hawaiian *Diplazium* based on six chloroplast regions – *atpA*, *atpB*, *matK*, *rbcL*, *rps4*+*rps4*–*trnS* IGS, and *trnL* intron+*trnL*–*trnF* IGS. We downloaded these plastid markers from GenBank for an additional 83 *Diplazium* species and two *Athyrium* species, and used the latter as outgroups. The resulting phylogeny inferred from combined data indicated that *D. arnottii* and *D. sandwichianum* are sister taxa, likely with origins in the Paleotropics. Morphologically, the two species can be distinguished by leaf dissection and lengths of sori. *Diplazium molokaiense* is sister to the Asian *D. heterocarpum* (among our sampled taxa). *Diplazium molokaiense* is distinguished from other species in the clade by its large sori and longer fronds. Our results reveal the phylogenetic placements of three *Diplazium* species and corroborate the placement of a fourth species.

Key Words.—eupolypods II, fern phylogeny, Hawaii, molecular phylogeny, plastid, Woodsiaceae

Hawaii is extremely isolated by distance from major land masses, leading to high levels of endemism. Of the 144 native fern species, 76% are endemic (Ranker, 2016). Ferns are found on every Hawaiian island and often create a dense, green ground covering, but some species are quite rare. Examples of these diverse ferns can be found in the genus *Diplazium* Sw., in which there are four Hawaiian species, three of which are endemic (Palmer, 2003; Vernon and Ranker, 2013).

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Diplazium is the largest genus in the lady-fern family Athyriaceae (Rothfels *et al.*, 2012b; PPG 1, 2016). With approximately 350–400 species, *Diplazium* has diverse morphologies and is found in a wide range of habitats (Christensen, 1906; Kato, 1977; Wei, Schneider, and Zhang, 2013). *Diplazium* has a pantropical distribution, with recently derived lineages located in tropical America after their ancestors migrated through the boreotropics from a likely origin in Eurasia (Wei *et al.*, 2015). Currently, approximately 70% of *Diplazium* species' diversity lies in Malesia, and 25% lies in the Neotropics (Tryon and Tyron, 1982; Pacheco and Moran, 1999; Mickel and Smith, 2004). The small remainder (<5%) of species can be found in Australia (Jones, 1998), New Zealand, many Pacific Islands, and the Afromadagascan region (Roux, 2009).

The taxonomic classification of the genus *Diplazium* has fluctuated greatly over time, with the circumscription of *Diplazium* further complicated by the recognition of some segregate genera such as *Allantodia* R. Br., *Callipteris* Bory, and *Monomelangium* Hayata with which *Diplazium* is often circumscribed (Chu and He, 1999; Wei, Schneider, and Zhang, 2013). *Diplazium* is a member of the Athyriaceae (Rothfels *et al.*, 2012a; Houser *et al.*, 2016; PPG 1, 2016). The broad range of morphologies among *Diplazium* species (Wei, Schneider, and Zhang, 2013) confounds efforts to infer a phylogeny based on morphological characters alone. Comparing frond shape and size is not sufficient, but species in *Diplazium* have distinctive patterns of soral shape and arrangement as well as scale color and shape (Wei, Schneider, and Zhang, 2013; Wei *et al.*, 2015). Four species of *Diplazium* can be found on the Hawaiian Islands: *Diplazium arnottii* Brack., *Diplazium esculentum* (Retz.) Sw., *Diplazium molokaiense* W.J. Rob., and *Diplazium sandwichianum* (C. Presl) Diels (Diels, 1899; Palmer, 2003; Ranker *et al.*, 2019).

Diplazium arnottii is endemic to Hawaii and can be found on the six largest islands (Palmer, 2003; Vernon and Ranker, 2013; Wood and Wagner, 2017). This species was first named *Asplenium diplazioides* (Hooker and Arnott, 1832), but it was moved to *Diplazium* by Brackenridge (1854). Like many other *Diplazium* species, *D. arnottii* is edible (Chock, 1968). *Diplazium arnottii* is often confused with *D. sandwichianum* in literature and during identification (Palmer, 2003). The two species often hybridize, and intermediates are common (Palmer, 2003). Little other knowledge is available for *D. arnottii*, including its taxonomic placement and evolutionary relationships with other fern species. The USDA (2014) suggests that *D. arnottii* is not overly abundant, but it is not considered endangered.

Diplazium esculentum is native to Southeast Asia and Indonesia. It was introduced to Hawaii sometime before 1910 (Wilson, 2002; Palmer, 2003), and it has spread to the six largest islands (Vernon and Ranker, 2013). Since that time, *D. esculentum* has become a part of local diets. Often, it is served as pohole salad at local markets. In the most recent maximum likelihood phylogeny of *Diplazium*, *D. esculentum* is grouped with *D. himalayense*, *D. giganteum*, and *D. viridissimum* with 100% bootstrap support (BS) (Wei and

Zhang, 2019). The sister taxon of *D. esculentum* was unclear (BS <70%; Wei, Schneider, and Zhang, 2013).

Diplazium molokaiense is an extremely rare fern that is endemic to Hawaii. It is listed as critically endangered on the IUCN Red List (Wood, 2006) and by the US Fish & Wildlife Service (Species Profile, 2020). We estimate that approximately 75 individuals remain (H. Oppenheimer, pers. obs.). At one time, *D. molokaiense* could be found on all major islands except Hawaii (Palmer, 2003), but the population has declined drastically. Currently, *D. molokaiense* can only be found in a Nature Conservancy site on Maui on a concave wall below overhanging rocks (Wood, 2006) and in volcanic fissures accessible only by helicopter and mountain hiking (pers. obs.). Their remote locations have made it difficult for researchers to access and study these populations. Even in the mid-19th century Hillebrand (1888) considered this fern to be very rare and noted its occurrence on Kauai, Oahu, and Molokai. Threats to the habitats in which *D. molokaiense* is found include destruction by feral animals (particularly pigs, cattle, and goats), predation by rats, flash floods, and competition with non-native plant taxa (Wood, 2006). Due to the rough terrain, the forest service cannot completely enclose the ferns in fencing to prevent animal interaction. Originally named *Asplenium arboreum* Hilleb. (Hillebrand, 1888), this species was reclassified as *D. molokaiense* in 1913 (Robinson, 1913).

Diplazium sandwichianum is a common fern in Hawaii. It is endemic to Hawaii and can be found on every main island except Kaho'olawe and Ni'ihau (Vernon and Ranker, 2013). *Diplazium sandwichianum* is an integral part of the native flora and makes up a large portion of the understory (Wood, 2006; Wood, 2011). Due to its great abundance, *D. sandwichianum* is often used to study plant litter decomposition (Allison and Vitousek, 2004a), plant litter nutrient cycling (Allison and Vitousek, 2004b), and the spread of invasive species, but its phylogenetic placement has never been estimated.

In this study, molecular data were used to address the following questions:

1. What is the phylogenetic placement of *D. arnottii, D. molokaiense*, and *D. sandwichianum*?
2. Do our results support the current position of *D. esculentum*?
3. From which geographic regions did Hawaiian *Diplazium* originate?

We hope that with collaboration from ecologists, conservation biologists, and systematists, this information can aid in the protection of Hawaii's native flora.

MATERIALS AND METHODS

Taxon sampling.—Fresh leaf tissue for the four *Diplazium* species was collected and identified by H. Oppenheimer from various nature preserves in Hawaii and silica-dried (Appendix 2).

TABLE 1. Primers used for PCR amplification and Sanger sequencing of six plastid regions in *Diplazium*.

Plastid marker	Primer Name	Sequence (5′–3′)
atpA	SJatpa-F	TGC TGA TGA GAA GAT TTA GC
	Satpa-R	TGG TTA TAA ACA TTC GAC CC
	SJatpa-IF*	GTT TGT AAA GGT TCG TAT GC
	SJatpa-IR*	TCT TTG CCA TTA AGA AGA CC
atpB	SJatpb-F	GAT CTT CTT CGG ATA ACT CG
	SJatpb-R	CGA AAT TTC TGA ATT GGT GG
	SJatpb-IF*	AAC ATC TCG AAA GTA TTC CG
	SJatpb-IR*	CTT TCG AGA TGT TAA CAA GC
matK	SJmatk-F	TAG TAG CGA AAT TGG AAT CC
	SJmatk-F	GAC ATT GGT CTA GTA TTC GG
rbcL	SJrbcl-F	ATT GCG TAT GTA GCT TAT CC
	SJrbcl-R	TCT TGC GTG AAA TAG ATA CC
rps4 + *rps4–trnS* IGS	SJrps4-F	GTC TTT CCA ACT AGA AAA CG
	SJrps4-R	CTC TCT ATT TCT ACC TCC C
trnL intron + *trnL–trnF* IGS	SJtrnl-F	TAT CTA AAT GTT TCG CGT GG
	SJtrnl-R	GAA TCC TAC GAT ATG TTG GG
	SJtrnl-IF*	AGT TCA AAT CTG GTT CTT GG
	SJtrnl-IR*	CGA ACA ATT TGT GTG AAT CC

* Indicates a primer only used for sequencing

DNA extractions, PCR, and DNA sequencing.—Total genomic DNA was extracted using the IBI Scientific Mini Genomic DNA Kit (Plant/Fungi) (Peosta, Iowa, USA) according to the manufacturer's instructions. Six chloroplast markers (*atpA, atpB, matK, rbcL, rps4+rps4–trnS* intergenic spacer [IGS], and *trnL intron+trnL–trnF* IGS), identified as informative in *Diplazium* by Wei, Schneider, and Zhang (2013) were the focus of PCR amplification and Sanger sequencing. PCR primers from the Wei, Schneider, and Zhang (2013) study did not successfully amplify all regions in our four species; therefore, new primers were designed (Table 1) based on the plastid genome of *Diplazium laffanianum* sequenced by Houser *et al.* (2016).

Chloroplast markers were amplified using PCR in 25 μL volumes containing 14.7 μL nuclease-free H_2O, 7.5 μL epicentre FailSafe PreMix Buffer B (Lucigen Corporation), 0.25 μL of a 20 μM solution of each forward and reverse primer, 0.25 μL *Taq* polymerase, and 2 μL of 1/50 unquantified DNA template. A dilution of 1/100 DNA template was used for *D. sandwichianum*. The PCR reaction conditions were as follows: one round of amplification consisting of denaturing at 96°C for 3 min, annealing at 45°C for 45 s, and extension at 72°C for 1 min, followed by 35 cycles of 94°C for 35 s, 45°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 12 min. Amplifications were visualized on 1% agarose gels with Lonza SYBR Green I Nucleic Acid Gel Stain and Thermo Scientific 6X DNA Loading Dye (Fisher Scientific). After running for 30 min at 100V, the gels were visualized with UV light. The amplified PCR products were cleaned using Exo-SAP by adding 2.25 μL nuclease-free H_2O, 0.25 μL of Exonuclease I (Life Technologies), and

0.50 μL of Shrimp Alkaline Phosphatase (Life Technologies) to each product. The solution was processed on a thermocycler at 37°C for 30 min, followed by 80°C for 15 min and held at 4°C for 30 min. PCR products were sequenced at the University of Nebraska Medical Center (UNMC) Core Sequencing Facility using Sanger sequencing.

Sequence alignment and phylogenetic analyses.—Sequences of all PCR products were assembled and edited in Geneious 10.2.6 (https://www.geneious.com). Sequences for the six plastid markers for an additional 83 closely related *Diplazium* species and two *Athyrium* (outgroup) species were downloaded from GenBank (Appendix 1; Houser *et al.*, 2016; Wei, Schneider, and Zhang, 2013). Sequences for each marker were aligned using MAFFT (v. 7.017; with default algorithm, scoring matrix: 200PAM / k = 2, gap open penalty: 1.53, and offset value 0.123; Katoh *et al.*, 2002 & 2013) in Geneious. Sequences were then manually adjusted to minimize gaps and correct misalignments. Since all six markers are from the chloroplast genome, they were combined into one data set for analysis. The final concatenated alignment was uploaded to the CIPRES Science Gateway (Miller, Pfeiffer, and Schwartz, 2010) for phylogenetic analyses.

The model of evolution was selected based on the Akaike information criterion (AIC) implemented in jModelTest2 on XSEDE (2.1.6) in CIPRES (www.phylo.org). jModelTest2 selected GTR+I+Γ as the best model of evolution for our data set. A maximum likelihood (ML) analysis was performed using RAxML-HPC v.8 on XSEDE (8.1.24) through CIPRES. Our data set was not partitioned for ML analysis. The likelihood score of the optimal tree that was generated by RAxML was calculated using PAUP* 4.0b10 (Swofford, 2003). ML nonparametric BS analyses were performed using RAxML-HPC v.8 on XSEDE (8.1.24) through CIPRES with 1000 replicates. A 50% majority rule consensus tree was constructed in PAUP*. Sequence alignments were uploaded to Dryad (www.datadryad.org).

RESULTS

Molecular.—Sequences were deposited into GenBank (Table 2). Sequences were not successfully obtained for every region for all species (marked with "–" in Table 2) and a few regions had internal gaps. The chloroplast markers that were successfully sequenced for each species varied. The greatest number of plastid markers (5 regions) were sequenced for *Diplazium molokaiense* and *D. sandwichianum*. The smallest number of regions (one + a partial) were sequenced for *D. arnottii*. Total aligned length was 6863 bp. The alignment is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.tht76hdwc.

The phylogeny of 89 species had high (>90%) to moderate (>75%) BS for most clades (Fig. 1). *Diplazium sandwichianum* and *D. arnottii* were sister taxa with strong support (BS=94%), forming a clade sister to *D. australe*, but with weak support (BS=57%). *Diplazium esculentum* was sister to the clade

TABLE 2. Lengths and GenBank accession numbers for four plastid genes (*atpA*, *atpB*, *matK*, and *rbcL*) and two intergenic spacers (*rps4-trnS* and *trnL-F*) for the four Hawiian *Diplazium* species.

Species		***atpA***	***atpB***	***matK***	***rbcL***	***rps4-trnS***	***trnL-F***
Diplazium arnottii	Length (bp)	–	–	857	–	–	1017
	Genbank Accession #	N/A	N/A	MT253574	N/A	N/A	MT253578
Diplazium esculentum	Length (bp)	871	1051	853	688	–	1114
	Genbank Accession #	MT253566	MT253570	MT253573	MT253576	N/A	MT253579
Diplazium molokaiense	Length (bp)	766	1110	867	686	923	1072
	Genbank Accession #	MT253567	MT253569	MT253575	MT253577	MT253583	MT253580
Diplazium sandwichianum	Length (bp)	1021	1114	747	–	762	1017
	Genbank Accession #	MT253568	MT253571	MT253572	N/A	MT253582	MT253581
Alignment length (bp)		1793	1411	1327	1308	2230	1384
Total length	6863 bp						

Note: "–" indicates the region was not sequenced. bp = base pairs

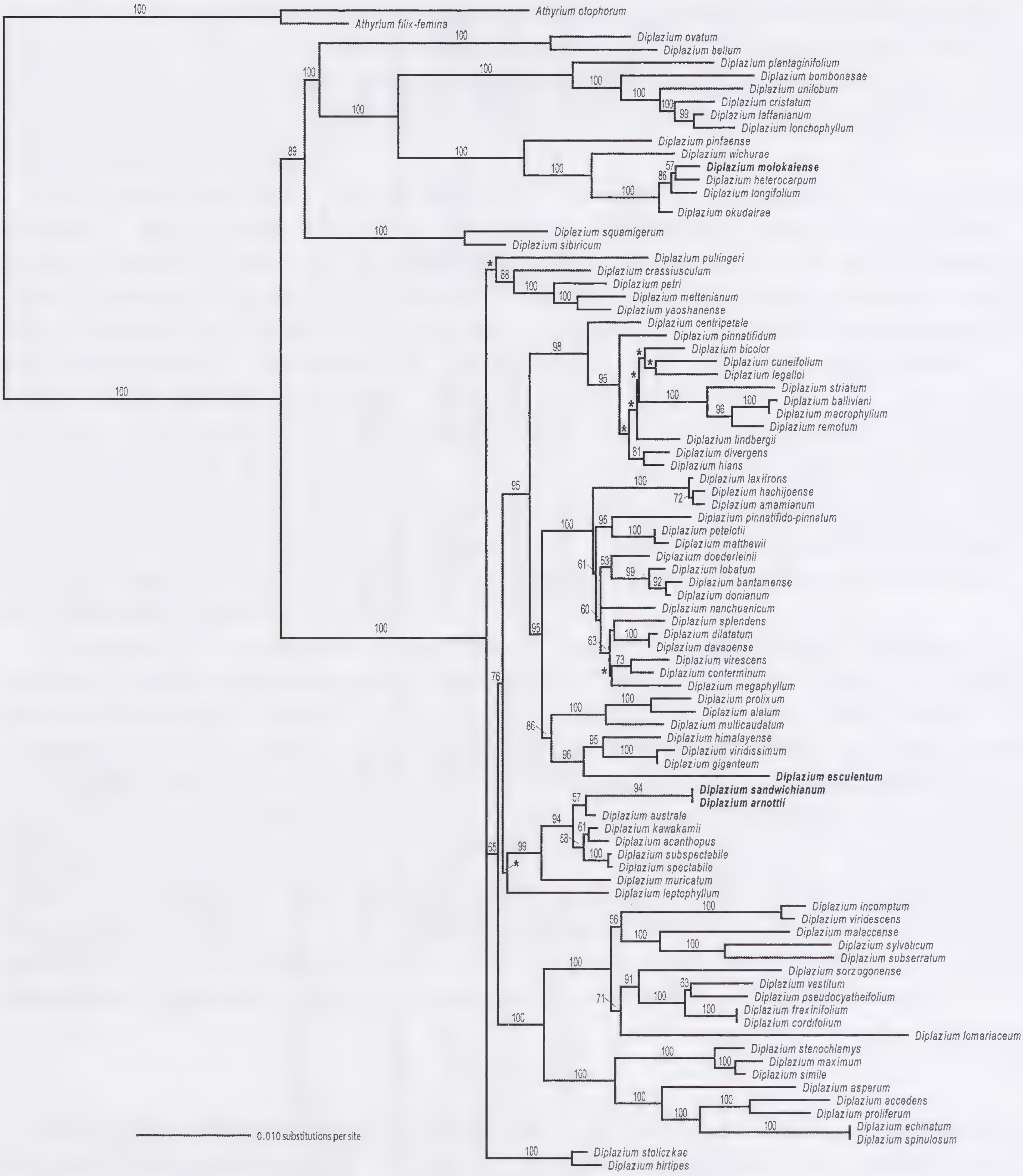

FIG. 1. Maximum likelihood (ML) tree (–ln L = 33962.483) for 87 species of *Diplazium* and two *Athyrium* species as outgroups, estimated from the concatenation of six chloroplast markers (*atpA*, *atpB*, *matK*, *rbcL*, *rps4*+*rps4–trnS* IGS, and *trnL* intron+*trnL–trnF* IGS). Numbers above branches indicate ML BS values resulting from 1000 replicates.

containing *D. himalayense, D. viridissimum*, and *D. giganteum* with strong support (BS=96%). *Diplazium molokaiense* was distantly related to the other three Hawaiian species. *Diplazium molokaiense* was sister to *D. heterocarpum* with weak support (BS=57%), but these species formed a clade with *D. longifolium* with moderate to strong support (BS=86%).

DISCUSSION

Our study provides evidence for the phylogenetic placements of Hawaii's four *Diplazium* species and identifies their putative closest relatives. *Diplazium arnottii, D. esculentum, D. molokaiense*, and *D. sandwichianum* are members of *Diplazium* with very strong (BS=100) support (Fig. 1). However, within *Diplazium*, these four species occur in three distinct and strongly supported clades, each containing many other *Diplazium* species that do not occur in Hawaii, suggesting at least two natural dispersal events to the seemingly isolated volcanic islands of Hawaii, not including the purposeful introduction of *D. esculentum.*

Among the Hawaiian species, *D. sandwichianum* and *D. arnottii* are most closely related. The two species often hybridize and individuals with intermediate characters are very common (Palmer, 2003). While typical forms of each species do exist, there is a full range of intermediate forms between the two extremes (Palmer, 2003). It is unclear if this array of morphologies is a result of incomplete speciation or complete speciation with subsequent introgressive hybridization (Palmer, 2003). The possibility of hybrids suggests that expanded sequencing may reveal additional evolutionary differences in the DNA.

Diplazium molokaiense is sister to *D. heterocarpum*, and this clade is sister to *D. longifolium* (Fig. 1). The phylogeny suggests a sister relationship between *D. molokaiense* and *D. heterocarpum*, but the support is weak (BS=57%). Morphologically, this relationship makes sense – all three species in this clade are 1-pinnate with oblong-lanceolate lamina, with shallowly to deeply crenate pinnae margins, an extended basal lobe on one side, reticulate venation, and double, linear sori (Beddome, 1883; Ching, 1936; Palmer, 2003). Despite the many similarities, *D. molokaiense* is distinguishable by its large sori, which can reach up to 1.5 cm in length (Palmer, 2003). *Diplazium heterocarpum* can be found only in China (GBIF *Diplazium heterocarpum*). *Diplazium molokaiense* is also part of a larger clade containing *D. okudairae* and *D. wichurae* (BS=100%). Both species are found in Japan, Korea, and China, with *D. wichurae* also found in Taiwan (Takamiya and Ohta, 2001). The two species share a similar environment to *D. molokaiense* and have very similar morphological characters (Johnson, 1986; Kato, 1977; Mettenius, 1899). It is possible that they are more closely related to *D. molokaiense* than *D. heterocarpum* or *D. longifolium*. Therefore, a molecular phylogeny inferred from additional chloroplast, mitochondrial, and nuclear ribosomal regions could lend support to evolutionary relationships.

The clade containing *Diplazium arnottii* and *D. sandwichianum* is sister to *D. australe* but with weak support (BS=57%). This position implies that *D. arnottii* and *D. sandwichianum* are the result of a single colonization event of an ancestral taxon from the Paleotropics (Wei, Schneider, and Zhang, 2013). *Diplazium australe* is found in Australia and New Zealand, just on the edge of the Paleotropics (GBIF *Diplazium australe*). The phylogenetic position of *D. molokaiense* (Fig. 1) suggests that the species is a result of a different colonization event – an ancestral migrant from Asia (Wei, Schneider, and Zhang, 2013). Our phylogeny supports the placement of *D. esculentum* published by Wei, Schneider, and Zhang (2013). This species was introduced by humans to Hawaii as opposed to arriving via a natural dispersal event. Most ancestors (59%) of today's Hawaiian ferns and fern allies likely originated in Asia or the Paleotropics, with the Pacific Rim contributing to a lesser extent (Ranker, 2016; Vernon and Ranker, 2013). A Jetstream causes a west-to-east wind that helps with dispersal of ancestral Hawaiian lineages from the Paleotropics and Asia (Fosberg, 1948; Carlquist, 1980; Geiger *et al.,* 2007; Ranker, 2016). From each colonizing ancestor, approximately 1.3 taxa are derived (Ranker, 2016). This is less than half of the approximate 3.6 derived species per colonizing ancestor for flowering plants (Wagner, Herbst, and Lorence, 2005; Ranker, 2016). Therefore, most genera that have multiple endemic species are the results of multiple, independent colonization events of ancestral taxa (Ranker, 2016).

Conclusions.—Our results can finally place three Hawaiian *Diplazium* fern species on the tree of life and reveal that there were likely at least two separate dispersal events to the islands. Most importantly, our results confirm the phylogenetic placement and endemic status of *D. molokaiense,* which is assessed as Critically Endangered on the IUCN red list. Additional research on the ecology, reproduction, and propagation of this species is needed, as well as further phylogenetic studies that incorporate more *Diplazium* species from around the world and additional molecular markers. With collaborative efforts from systematists, ecologists, conservation biologists, and additional field researchers, we can aid in the protection of Hawaii's native species.

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Appendix 1. GenBank accession numbers for four plastid genes (*atpA, atpB, matK,* and *rbcL*) and two intergenic spacers (*rps4-trnS* and *trnL-F*) for the species included in the sequence alignment. Dashes indicate an unavailable sequence.

Species	*atpA*	*atpB*	*matK*	*rbcL*	*rps4-trnS*	*trnLF*
A. filix-femina	JF832096[e]	JF832154[e]	JF303941[d]	JF832056[e]	AF425152[g]	EU329076[a]
A. otophroum	JF832098[e]	EF463563[f]	JF832258[e]	EF463305[f]	JN168076[b]	AF515236[i]
D. acanthopus	KC254238[j]	KC254317[j]	KC254158[j]	KC254391[j]	KC254546[j]	KC254466[j]
D. accedens	KC254229[j]	KC254308[j]	KC254150[j]	KC254383[j]	KC254537[j]	KC254457[j]
D. alatum	KC254250[j]	KC254329[j]	KC254171[j]	KC254402[j]	KC254558[j]	KC254480[j]
D. amamianum	KC254258[j]	KC254337[j]	KC254179[j]	KC254410[j]	KC254566[j]	KC254488[j]
D. asperum	KC254224[j]	KC254303[j]	KC254145[j]	KC254379[j]	KC254531[j]	KC254451[j]
D. australe	KC254239[j]	KC254318[j]	KC254159[j]	KC254392[j]	–	KC254467[j]
D. ballivianii	–	–	KC254169[j]	–	KC254556[j]	KC254478[j]
D. bantamense	KC254271[j]	–	KC254192[j]	KC254422[j]	KC254579[j]	KC254501[j]
D. bellum	KC254200[j]	KC254279[j]	KC254121[j]	KC254356[j]	KC254507[j]	KC254428[j]
D. bicolor	KC254243[j]	KC254322[j]	KC254163[j]	KC254396[j]	KC254549[j]	KC254471[j]
D. bombonasae	EF463911[f]	EF463570[f]	JF832273[e]	EF463308[f]	–	KX656171[d]
D. centripetale	EF463912[f]	EF463571[f]	–	EF463309[f]	–	–
D. conterminum	KC254265[j]	KC254344[j]	KC254186[j]	KC254416[j]	KC254573[j]	KC254495[j]
D. cordifolium	KC254220[j]	KC254299[j]	KC254141[j]	–	KC254527[j]	KC254448[j]
D. crassiusculum	KC254214[j]	KC254293[j]	KC254135[j]	KC254370[j]	KC254521[j]	KC254442[j]
D. cristatum	KC254206[j]	KC254285[j]	KC254127[j]	KC254362[j]	KC254515[j]	KC254434[j]
D. cuneifolium	KC254248[j]	KC254326[j]	KC254168[j]	–	KC254554[j]	KC254476[j]
D. davaoense	KC254268[j]	KC254347[j]	KC254189[j]	KC254419[j]	KC254576[j]	KC254498[j]
D. dilatatum	KC254267[j]	KC254346[j]	KC254188[j]	KC254418[j]	KC254575[j]	KC254597[j]
D. divergens	KC254244[j]	KC254323[j]	KC254164[j]	KC254397[j]	KC254550[j]	KC254472[j]
D. doederleinii	KC254260[j]	KC254339[j]	KC254181[j]	KC254412[j]	KC254568[j]	KC254490[j]
D. donianum	KC254272[j]	KC254349[j]	KC254193[j]	KC254423[j]	KC254580[j]	KC254502[j]
D. echinatum	KC254226[j]	KC254305[j]	KC254147[j]	KC254381[j]	KC254533[j]	KC254453[j]
D. fraxinifolium	–	–	–	–	KC254536[j]	KC254456[j]
D. giganteum	KC254252[j]	KC254331[j]	KC254173[j]	KC254404[j]	KC254560[j]	KC254482[j]
D. hachijoense	KC254256[j]	KC254335[j]	–	KC254408[j]	KC254564[j]	KC254486[j]
D. heterocarpum	KC254203[j]	KC254282[j]	KC254124[j]	KC254359[j]	KC254510[j]	KC254431[j]
D. hians	KC254245[j]	KC254324[j]	KC254165[j]	KC254398[j]	KC254551[j]	KC254473[j]
D. himalayense	KC254255[j]	KC254334[j]	KC254176[j]	KC254407[j]	KC254563[j]	KC254485[j]
D. hirtipes	KC254211[j]	KC254290[j]	KC254132[j]	KC254367[j]	KC254518[j]	KC254439[j]
D. incomptum	KC254218[j]	KC254297[j]	KC254139[j]	KC254374[j]	KC254525[j]	KC254446[j]
D. kawakamii	KC254236[j]	KC254315[j]	KC254157[j]	KC254390[j]	KC254544[j]	KC254464[j]
D. laxifrons	KC254257[j]	KC254336[j]	KC254178[j]	KC254409[j]	KC254565[j]	KC254487[j]
D. legalloi	EF463916[f]	EF463575[f]	–	EF463313[f]	–	–
D. leptophyllum	–	KC254319[j]	KC254160[j]	KC254393[j]	–	KC254468[j]
D. lindbergii	KC254246[j]	–	KC254166[j]	KC254399[j]	KC254552[j]	KC254474[j]
D. lobatum	KC254270[j]	–	KC254191[j]	KC254421[j]	KC254578[j]	KC254500[j]
D. lomariaceum	KC254221[j]	KC254300[j]	KC254142[j]	KC254375[j]	KC254528[j]	KC254449[j]
D. lonchophyllum	–	–	–	U05920[l]	–	–
D. longifolium	KC254204[j]	KC254283[j]	KC254125[j]	KC254360[j]	KC254511[j]	KC254432[j]
D. macrophyllum	KC254247[j]	KC254325[j]	KC254167[j]	KC254400[j]	KC254553[j]	KC254475[j]
D. malaccense	KC254228[j]	KC254307[j]	KC254149[j]	–	KC254535[j]	KC254455[j]
D. matthewii	KC254263[j]	KC254342[j]	KC254184[j]	KC254415[j]	KC254571[j]	KC254493[j]
D. maximum	KC254231[j]	KC254310[j]	KC254152[j]	KC254385[j]	KC254539[j]	KC254459[j]
D. megaphyllum	KC254264[j]	KC254343[j]	KC254185[j]	–	KC254572[j]	KC254494[j]
D. mettenianum	KC254212[j]	KC254291[j]	KC254133[j]	KC254368[j]	KC254519[j]	KC254440[j]
D. multicaudatum	KC254251[j]	KC254330[j]	KC254172[j]	KC254403[j]	KC254559[j]	KC254481[j]

Appendix 1. Continued.

Species	*atpA*	*atpB*	*matK*	*rbcL*	*rps4-trnS*	*trnLF*
D. muricatum	KC254235[j]	KC254314[j]	KC254156[j]	KC254389[j]	KC254543[j]	KC254463[j]
D. nanchuanicum	KC254259[j]	KC254338[j]	KC254180[j]	KC254411[j]	KC254567[j]	KC254489[j]
D. okudairae	KC254201[j]	KC254280[j]	KC254122[j]	KC254357[j]	KC254508[j]	KC254429[j]
D. ovatum	KC254199[j]	KC254278[j]	KC254120[j]	KC254355[j]	KC254506[j]	KC254427[j]
D. petelotii	KC254262[j]	KC254341[j]	KC254183[j]	KC254414[j]	KC254570[j]	KC254492[j]
D. petrii	KC254213[j]	KC254292[j]	KC254134[j]	KC254369[j]	KC254520[j]	KC254441[j]
D. pinfaense	KC254202[j]	KC254218[j]	KC254123[j]	KC254362[j]	KC254509[j]	KC254430[j]
D. pinnatifido-pinnatum	KC254261[j]	KC254340[j]	KC254182[j]	KC254413[j]	KC254569[j]	KC254491[j]
D. pinnatifidum	KC254241[j]	KC254320[j]	KC254161[j]	KC254394[j]	–	KC254469[j]
D. plantaginifolium	KC254205[j]	KC254284[j]	KC254126[j]	KC254361[j]	KC254514[j]	KC254433[j]
D. proliferum	EF463918[f]	EF463577[f]	JF303939[c]	EF463315[f]	–	KX656173[d]
D. prolixum	KC254249[j]	KC254328[j]	KC254170[j]	KC254401[j]	KC254557[j]	KC254479[j]
D. pseudocyanthei-folium	KC254222[j]	KC254301[j]	KC254143[j]	KC254377[j]	KC254529[j]	KC254450[j]
D. pullingeri	KC254209[j]	KC254288[j]	KC254130[j]	KC254364[j]	KC254516[j]	KC254437[j]
D. remotum	–	KC254327[j]	–	–	KC254555[j]	KC254477[j]
D. sibiricum	KC254208[j]	KC254287[j]	KC254129[j]	KC254364[j]	KC254513[j]	KC254436[j]
D. simile	KC254230[j]	KC254309[j]	KC254151[j]	KC254384[j]	KC254538[j]	KC254458[j]
D. sorzogonense	KC254223[j]	KC254302[j]	KC254144[j]	KC254378[j]	KC254530[j]	–
D. spectabile	KC254234[j]	KC254313[j]	KC254155[j]	KC254388[j]	KC254542[j]	KC254462[j]
D. spinulosum	KC254225[j]	KC254304[j]	KC254146[j]	KC254380[j]	KC254532[j]	KC254452[j]
D. splendens	KC254269[j]	KC254348[j]	KC254190[j]	KC254420[j]	KC254577[j]	KC254499[j]
D. squamigerum	KC254207[j]	KC254286[j]	KC254128[j]	KC254363[j]	KC254512[j]	KC254435[j]
D. stenochlamys	KC254232[j]	KC254311[j]	KC254153[j]	KC254386[j]	KC254540[j]	KC254460[j]
D. stoliczkae	KC254210[j]	KC254289[j]	KC254131[j]	KC254366[j]	KC254517[j]	KC254438[j]
D. striatum	KC254242[j]	KC254321[j]	KC254162[j]	KC254395[j]	KC254548[j]	KC254470[j]
D. subserratum	KC254219[j]	KC254298[j]	KC254140[j]	KC254375[j]	KC254526[j]	KC254447[j]
D. subspectabile	KC254233[j]	KC254312[j]	KC254154[j]	KC254387[j]	KC254541[j]	KC254461[j]
D. sylvaticum	KP318887[k]	KP318898[k]	KP318907[k]	KP318918[k]	KP318929[k]	KP318940[k]
D. unilobum	KP318893[k]	–	KP318913[k]	KP318924[k]	KP318934[k]	KP318946[k]
D. vestitum	KC254227[j]	–	KC254148[j]	KC254382[j]	KC254534[j]	KC254454[j]
D. virescens	KC254266[j]	KC254345[j]	KC254187[j]	KC254417[j]	KC254574[j]	KC254496[j]
D. viridescens	KC254217[j]	KC254296[j]	KC254138[j]	KC254373[j]	KC254524[j]	KC254445[j]
D. viridissimum	KC254253[j]	KC254332[j]	KC254174[j]	KC254405[j]	KC254561[j]	KC254483[j]
D. wichurae	JF832117[e]	EF463579[f]	JF832275[e]	AB042743[h]	AF515245[i]	–
D. yaoshanense	KC254215[j]	KC254294[j]	KC254136[j]	KC254371[j]	KC254522[j]	KC254443[j]

[a] Adjie *et al.*, 2008

[b] Chunxiang *et al.*, 2011

[c] Kuo *et al.*, 2011

[d] Kuo *et al.*, 2016

[e] Rothfels *et al.*, 2012b

[f] Schuettpelz and Pryer, 2007

[g] Smith and Cranfill, 2002

[h] Takamiya *et al.*, 2001

[i] Wang *et al.*, 2003

[j] Wei, Schneider, and Zhang, 2013

[k] Wei et al., 2015

[l] Wolf, Soltis, and Soltis, 1994

APPENDIX 2. Voucher information for *Diplazium arnottii, Diplazium esculentum, Diplazium molokaiense*, and *Diplazium sandwichianum*. All specimens were collected and identified by Hank Oppenheimer.

Species	**Collection Location**	**Date**	**Collection Number**	**Herbarium of Deposition**
Diplazium arnottii	Palikea Stream, Maui	9 Aug, 2017	H81714	HALE
Diplazium esculentum	Wailua, east Maui	9 Oct, 2017	Field ID by H. Oppenheimer	
Diplazium molokaiense	Kula Forest Reserve, Maui	22 Aug, 1998	H89802	BISH
Diplazium sandwichianum	Kula Forest Reserve, Maui	3 Oct, 2018	H101801	OMA

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Polycyclic solenostele–a new synapomorphy for *Pteris* sect. Litobrochia

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ABSTRACT.—The vascular architecture of the stem–the stele–is one of the most well studied topics in pteridology, and has fascinated botanists for more than 200 years. Stelar architecture has attracted much attention, in part, due to its diversity in the fern lineage as well as its use as a stable taxonomic character for circumscribing species, genera, families, or larger informal clades. One aspect of stelar morphology that has garnered specific attention is polystely: the development of multiple concentric cylinders of vasculature. This feature has evolved in lineages throughout the fern phylogeny, including in the genus *Pteris* L. (Pteridaceae). Here, I describe a polycyclic solenostele as a new synapomorphy for *Pteris* sect. Litobrochia. The identification of this character as a synapomorphy furthers our understanding of the morphological evolution and circumscription of *Pteris* as well as aides in species identification within this speciose genus.

KEYWORDS.—stele; rhizome; vasculature; morphology; ancestral character state estimation; Pteridaceae

The stele is defined as the primary vascular system of the stem and is generally not used to describe the vasculature of the petiole, root, or reproductive structures (Schmid, 1982). There are three broad categories of stelar architecture: protosteles, solenosteles, and dictyosteles–all varying in characters like the presence of a pith, and the arrangements of leaf gaps. The categorization, development, evolution, and taxonomic importance of stelar architecture has interested pteridologists for over two centuries (Van Tieghem and Douliot, 1886; Gwynne-Vaughan, 1903; Tansley, 1908). In particular, stelar morphology has been a focal point in pteridology due to its diversity and use as a systematic tool for circumscription (Bower, 1923; Ogura, 1972; Mitsuta, Kato, and Iwatsuki, 1980; Schmid, 1982; Becari-Viana and Schwartsburd, 2017).

The development of concentric vascular cylinders, polystely (Fig. 1a–c), has attracted attention in certain fern lineages. Polycyclic steles are found in taxa scattered throughout the fern phylogeny including the Matoniaceae, Dennstaedtiaceae, Pteridaceae, and the Saccolomataceae (Bower, 1928). While this character state appears in a broad set of taxa throughout ferns, it is found in fewer than a few dozen species (Bower, 1928; Ogura, 1972)–for this reason it has been used as a taxonomic tool for identification (Tansley, 1907c). Despite dedicated study on stelar morphology during the early 20th century, phylogenetic relationships between some of the families possessing species with polycyclic steles were not well resolved (Pryer, Smith, and Skog, 1995; Pryer *et al.*, 2004; Schuettpelz and Pryer, 2007; Rothfels *et al.*, 2015). This led to uncertainty in properly ascribing stelar architecture to taxonomic groups.

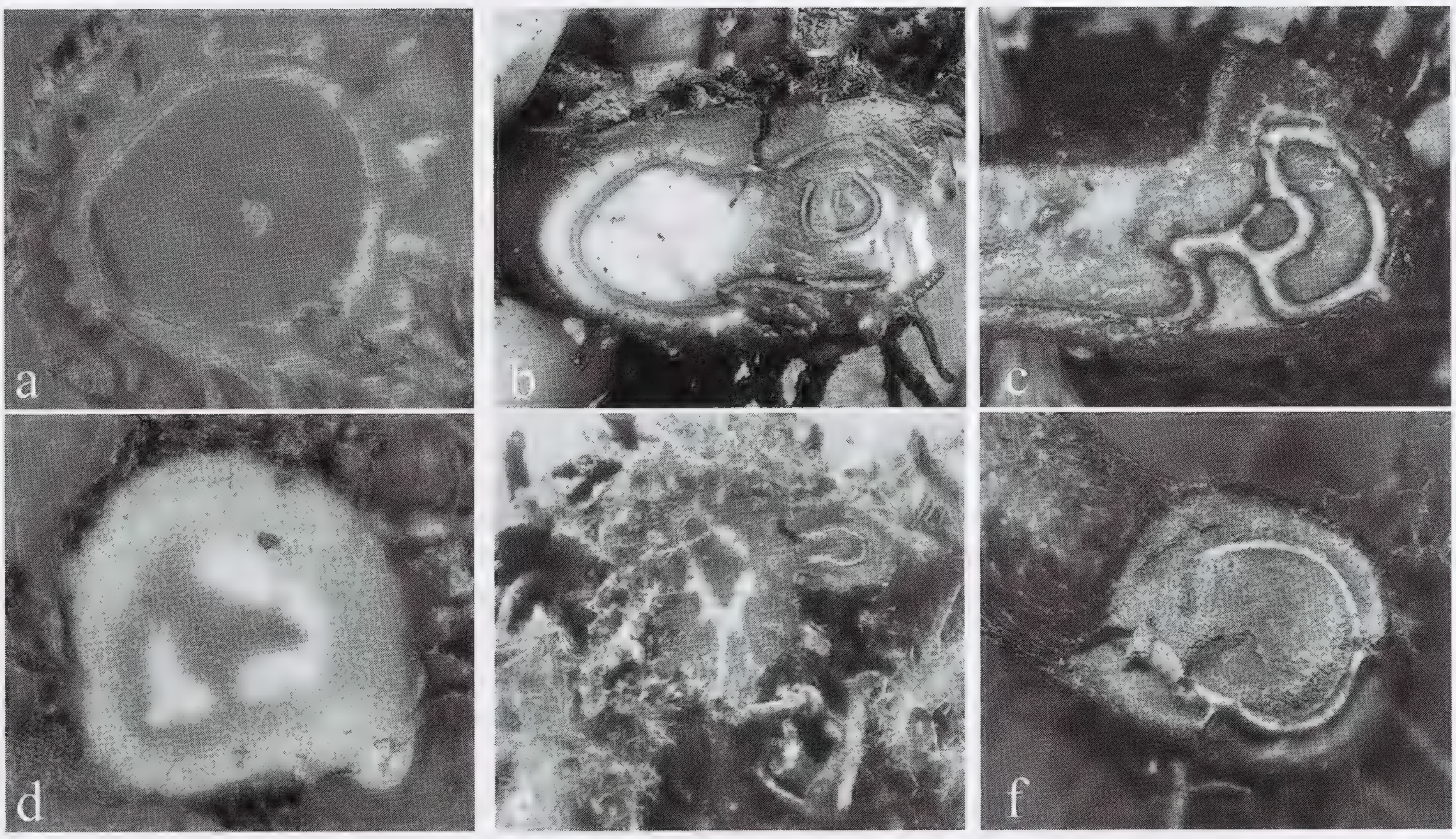

Fig. 1. a. Polycyclic solenostele, *Pteris podophylla,* inner stele initiating; b. Polycyclic solenostele, *Pteris livida*: Photo courtesy of Robbin Moran; c. Polycyclic solenostele, *Pteris muricate*: Photo courtesy of Weston L. Testo; d. Simple solenostele-dictyostele, *Pteris ensiformis;* e. Simple solenostele-dictyostele, *Pteris plumula*; f. Simple solenostele-dictyostele, *Pteris papuana*. Photo courtesy of Michael A. Sundue.

Specifically, within the Pteridaceae the monophyly of the genus *Pteris* has been questioned for some time (Chao *et al.*, 2014; Zhang *et al.*, 2015; Zhang and Zhang, 2018). Circumscription of this genus has been difficult, in part, due to the large number of species and morphological diversity. The genus is comprised of ca. 250 species that are globally distributed and grow in habitats varying from rocky outcrops to moist forest understories (Tryon, 1990). The species richness and ecological diversity in the genus are mirrored by their morphological diversity. Leaf size, rhizome form, and vein morphology vary throughout the genus (Tryon, 1990; Zhang and Zhang, 2018). Recent work has aimed to resolve the phylogeny, biogeography, and character evolution in the genus (Chao *et al.*, 2014; Zhang *et al.*, 2015; Zhang and Zhang, 2018; Chao and Huang, 2018). Here, I contribute to the morphological circumscription and evolution of the genus *Pteris* by describing a new synapomorphy for sect. Litobrochia. By combining morphology with recent phylogenies and phylogenetic comparative methods, I reveal that the polycyclic solenostele evolved independently in *Pteris* sect. Litobrochia and is a defining feature of this clade that can be used to assist in species identification.

MATERIALS AND METHODS

Morphological data used in this study were obtained from herbarium specimens in the HUH or from the literature (Table 1). Species were chosen in

TABLE 1. Species used in this analysis with the states of stelar architecture and the reference for ID. All vouchered specimens were from the HUH. All specimens documented as cultivated were living in the Weld Hill Research Center Growth Facilities at the Arnold Arboretum of Harvard University. When multiple sources are present for the same species, they are documented and separated by a semicolon.

Taxa	State	Voucher	Other reference
Pteris chiapensis A. R. Sm.	Polycyclic solenostele	Breedlove 24492	
Pteris wallichiana C. Presl	Polycyclic solenostele		Chandra and Nayar, 1970
Pteris orizabae M. Martens & Galeotti	Polycyclic solenostele	GBH 3519; Tryon and Tryon 305	Plantsystematics.org
Pteris deflexa Link	Polycyclic solenostele	EBC 4853	
Pteris haenkeana C. Presl	Polycyclic solenostele	Wade Davis 1134	
Pteris speciosa Kuhn	Polycyclic solenostele	Madison 789	
Pteris decurrens C. Presl	Polycyclic solenostele	Meyer 9350; Solbrig 3828	
Pteris altissima Poir.	Polycyclic solenostele		Ogura, 1972
Pteris podophylla Sw.	Polycyclic solenostele	Ollgaard and Balslev 9285	Bower, 1923
Pteris livida Mett.	Polycyclic solenostele		Plantsystematics.org
Pteris muricata Hook	Polycyclic solenostele	Leon Fernandez and Silva 948	Fernsoftheworld.com
Pteris vieillardii Mett.	Solenostele	1861-67	
Pteris venulosa Blume	Solenostele	AR 13	
Pteris fauriei Hieron.	Solenostele	Bartholomew Sino-American Guizhou Bot Exp 29; Taihoku Syu 17798	
Pteris longipes D. Don	Solenostele	Bartsch 514	
Pteris vittata L.	Solenostele	Boufford and Bartholomew 24001; WT Tsang 23646	
Pteris bella Tagawa	Solenostele	Boufford and Bartholomew 25192	
Pteris pellucida C. Presl	Solenostele		Bower, 1928
Onychium japonicum (Thunb.) Kunze	Solenostele		Bower, 1928
Taenitis blechnoides (Willd.) Sw.	Solenostele		Bower, 1928
Pteris dentata Forssk.	Solenostele		Bower, 1928
Pteris biaurita L.	Solenostele	Fleming 865; Bievre 5800; Congdon 1211	Bower, 1928

TABLE 1. Continued.

Taxa	State	Voucher	Other reference
Pteris grandifolia Blanco	Solenostele	Mexia 6182	Bower, 1928
Pteris cretica L.	Solenostele		Bower, 1928; Ogura, 1972
Pteris ryukyuensis Tagawa	Solenostele	CED 8357	
Pteris grevilleana Wall.	Solenostele	Chi-Cheng Liao 1121	
Pteris cadieri Christ	Solenostele	Chi-Cheng Liao 798	
Pityrogramma trifoliata (L.) R.M. Tryon	Solenostele	Correll and Smith P913; Dudley 11729; Ynex Mexia 04100	
Pteris aspericaulis Wall.	Solenostele	Dickason 7571; Dickason 7210; Iwatsuki *et al.* 168; GLGS 18971; Iwatsuki 1050	
Pteris bahamensis Fée	Solenostele	Eaton 931	
Pteris macilenta A. Rich.	Solenostele	Prince 1898	
Pteris setulosocostulata Hayata	Solenostele	Feung and Kao 222	
Pteris aspericaulis Wall.	Solenostele	Heng 18971; Iwatsuki 1050	
Pteris leptophylla Sw.	Solenostele	434 Kew	
Pteris actiniopteroides Christ.	Solenostele	Bartholomew 27	
Pteris lechleri Mett.	Solenostele	Dudley 11946; Killip and Smith 23962	
Pteris splendens Kaulf.	Solenostele	EBC 4931; Handro 2221	
Pteris dactylina Hook.	Solenostele	JF Rock 4880	
Pteris pungens Willd.	Solenostele	M. E. Peck 628	
Pteris burtonii Baker	Solenostele	OF Cook 80	
Pteris denticulate Sw.	Solenostele	Rojas 12915; Burkart 1535	
Pteris henryi Christ	Solenostele	SP Ko 55789	
Pteris multifida Poir.	Solenostele	Tsang W. T. 27721	
Pteris buchananii Bak.ap.Sim	Solenostele	Tyon 6391	
Pteris quadriaurita Retz.	Solenostele	Biswas 3853	

Table 1. Continued.

Taxa	State	Voucher	Other reference
Pteris praestantissima (Fée) Christenh.	Solenostele		Plantsystematics.org
Pteris linearis Wall.	Solenostele	Hu and But 21079	
Pteris umbrosa R.Br.	Solenostele	Johnson and Constable P9771	
Pteris spinescent C. Presl	Solenostele	Kramer and Gassner 7719	
Pterozonium brevifrons (A.C.Sm.) Lellinger	Solenostele		Kramer and Green, 1990
Austrogramme decipiens (Mett.) Hennipman	Solenostele		Kramer and Green, 1990
Syngramma quinata (Hook.) Carruth.	Solenostele		Kramer and Green, 1990
Anogramma leptophylla (L.) Link	Solenostele		Kramer and Green, 1990
Pteris terminalis Wall.	Solenostele	LF Brass 22939	
Pteris nipponica Shieh	Solenostele	Liu 523	
Pteris tripartite Sw.	Solenostele	LJ Brass 25438	
Pteris chilensis Desv.	Solenostele	Meyer 9424	
Pteris papuana Ces.	Solenostele		Fernsoftheworld.com
Pteris moluccana Blume	Solenostele	Miller and Holttum 18636	
Pteris oshimensis Hieron	Solenostele	Mitsuta 12484	
Pteris longipinnula Wall.	Solenostele	Molesworth 3434	
Pteris tremula R.Br.	Solenostele		Ogura, 1972
Pteris brasiliensis Raddi	Solenostele	Palacios-Cuezzo 736	
Pteris ensiformis Burm.	Solenostele		Cultivated
Actiniopteris semiflabellata Pic.Serm.	Solenostele		Cultivated
Pteris heteromorpha Fée	Solenostele	Petrmitr 385	

Table 1. Continued.

Taxa	State	Voucher	Other reference
Pteris insignis Mett.	Solenostele	Phengklai 3525	
Pteris venusta Kunze	Solenostele	Phuakam 11	
Pteris praetermissa T.G.Walker	Solenostele		Plantsystematics.org
Pteris nervosa Thunb.	Solenostele	RC Ching 75079	Bower, 1928
Pteris scabripes Wall.	Solenostele	Sands MJS 6701	
Pteris comans G.Forst.	Solenostele	Solbrig 3828; Meyer 9350	
Pteris subquinata Wall.	Solenostele	Suzuki 9485138	
Pteris decrescens Christ	Solenostele	Tagawa 2370 Thailand; Phankan 3; Chamsai 108	
Pteris pacifica Hieron.	Solenostele	Takeuchi 9277	
Pteris xichouensis W.M.Chu & Z.R.He	Solenostele		Tropicos.org synonym (*P. cretica*)
Jamesonia flexuosa (Humb. & Bonpl) Christenh.	Solenostele	Tryon 1990	
Pteris friesii Hieron.	Solenostele	Tryons 6228	
Pteris catoptera Kunze	Solenostele	Tryons 6435	
Pteris kiuschiuensis Hieron.	Solenostele	Tsang 20975	
Pteris semipinnata L.	Solenostele	Tsang WT 29074; Tsang WT 29934	
Pteris khasiana (C.B.Clarke) Hieron.	Solenostele	van der Werff 23941	
Pteris usambarensis Hier.in.Engl	Solenostele	Verdcourt 310	
Pteris gallinopes Ching	Solenostele	Wang 75846	
Pteris platyzomopsis Christenh. & H. Schneid	Solenostele	White 9705 1934	
Pteris excelsa Gaudich.	Solenostele	WP 2006	
Pteris deltodon Baker	Solenostele	WP 605 Vietnam; WP 690 Vietnam	
Pteris dissitifolia Baker	Solenostele	WT Tsang 29943; WT Tsand 30327	

TABLE 1. Continued.

Taxa	State	Voucher	Other reference
Pteris morii Masam.	Solenostele	Wuzhishan Fern survey 272	
Pteris mutilate L.	Solenostele	Wuzhishan Fern survey 595	
Pteris hainanensis (Ching) S.H.Wu	Solenostele	Wuzhishan Fern Survey 607	
Pteris longifolia Cav.	Solenostele	Ynes Mexia 8942a	
Pteris propinqua J.Agardh	Solenostele	Bennett 9	

this study based on their presence in the maximum likelihood (ML) phylogeny of *Pteris* and its outgroups in Zhang and Zhang (2018). For information on the phylogenetic estimation see Zhang and Zhang (2018). In short, a ML phylogeny was estimated for 256 accessions, representing ~178 species of *Pteris*, from seven plastid markers (*atpA, rbcL, rps4-trnS, trnL, trnL-F, and trnL & trnL-F*) and one nuclear marker (*gapcp*). The single ML phylogeny that corresponds to Supplementary data 1 in Zhang and Zhang (2018) was used for all downstream analyses here. If species in the phylogeny had an herbarium specimen with an attached rhizome, stelar morphology was visualized without destructive sampling, when possible. When specimens did not have mounted rhizomes, they were excluded from the dataset. A data matrix containing species name, stelar morphology, and voucher number was generated (Table 1). Stelar morphology of species that were described in the literature were used to supplement the data.

In order to use model-based methods of ancestral character state estimation, a phylogeny with branch lengths in particular units is needed because these are required when calculating the transition rates; it is best practice to have the branch lengths in units of absolute time (O'Meara, 2012). To generate a time calibrated phylogeny, three nodes were secondarily calibrated using upper and lower bounds from a recent time calibrated fern phylogeny (Testo and Sundue, 2016). These nodes correspond to the crown group of *Pteris* (61.64–79.78my), the most recent common ancestor of *Onychium* and *Pteris* (80.13–98.41my), and the crown group of the pteridoid clade (121.88–128.21my). These calibration points were applied using the *chronos* function in APE (Paradis and Schliep, 2018). These data were analyzed under a relaxed, strict, and correlated clock model–the relaxed clock model best fit the data according to the information criterion outlined by Sanderson (2002). A relaxed clock model with 10 rate categories and a smoothing parameter of 0 was used for calibration; this model corresponds to the saturated (SAT) model (Sanderson, 2002), which allows each branch of the phylogeny to have a different rate of molecular evolution (Paradis, 2013).

To visualize the evolution of stelar morphology in *Pteris*, stochastic character mapping was implemented using the function *make.simmap* in

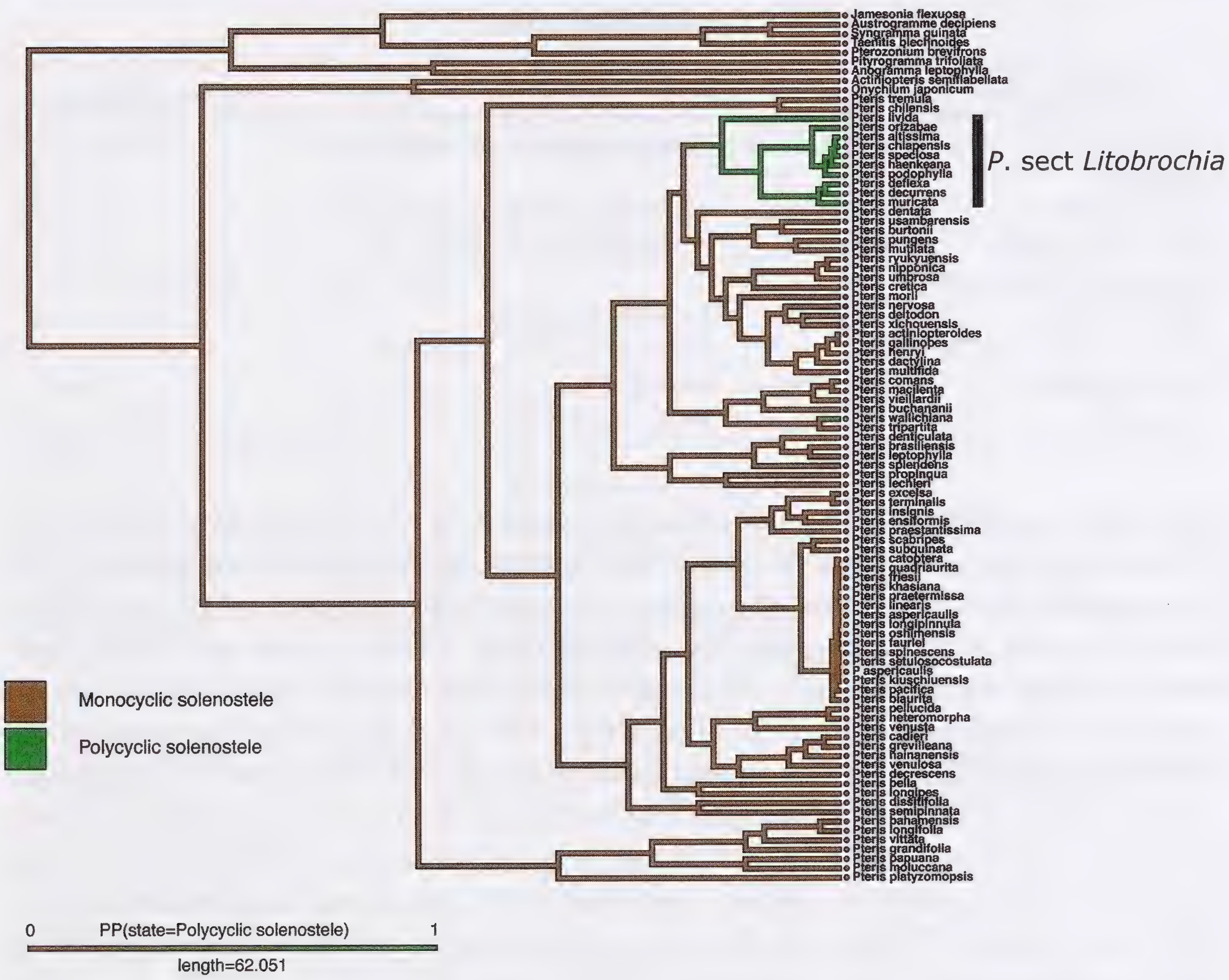

Fig. 2. Ancestral character state estimation using stochastic character mapping. 1000 simulated character histories are summarized by plotting the posterior density for being in a polycyclic solenostelic state on the edges of the phylogeny.

phytools (Revell, 2012). This approach implements the stochastic character mapping algorithm by Bollback (2006). Ancestral character states were estimated under the best fitting model (equal transition rates) as determined by Akaike information criterion (AIC). One thousand character histories were simulated on the one ML phylogeny described above (Fig. S1). Posterior probabilities of each state at each node were plotted on the ML phylogeny (Fig. S2). The 1,000 simulated character histories were summarized to generate a tree colored by the posterior probability density for being in the polycyclic solenostelic state using the function *densityMap* in phytools (Revell, 2012) (Fig. 2).

RESULTS

The ancestral estimation of stelar morphology in the pteridoid clade is reconstructed as a monocyclic solenostele. There are two transitions to polycycly, one at the base of the clade leading to *Pteris* sect. Litobrochia and the other is along the branch leading to *P. wallichiana* (Fig. 2). It should be noted that some species in *Pteris* possess a monocyclic solenostele where leaf gaps will overlap or not depending on the size of leaf gaps and internode length (Gwynne-Vaughan, 1903; Bower, 1923). A cross section through one slice of the rhizome may not reveal the entire architecture throughout the rhizome, but it will determine whether a species is monocyclic or polycyclic. Because leaf gaps can overlap in some but not all parts of an individual rhizome it can be transitionary within an individual; a determination of this transitionary type was not specifically coded here.

Within *Pteris* a monocyclic solenostele is almost ubiquitous (Fig 1d–f; Fig. 2). All species observed in *Pteris* sect. Litobrochia possess a polycyclic solenostele (Fig. 1a–c; Fig. 2), which represents an independent origin of this stelar architecture in this clade. The stelar morphology of species in *P.* sect Litobrochia consists of a larger outer cylinder with leaf gaps, and an inner cylinder which seems to perforate arbitrarily–although a 3-dimensional reconstruction may provide insight to the correlation of gaps in the inner bundle. The leaf gaps of the outer bundles do not overlap with frequency, although upon occasion they may. For this reason, this stele is termed a polycyclic solenostele, instead of a polycyclic dictyostele, which would indicate consistently overlapping leaf gaps. The inner bundle seems to fuse with the outer bundle following leaf trace departure (Fig. 1c).

The rhizomes of species in *P.* sect Litobrochia are robust compared to many other members of the genus (Tryon, 1990; Zhang and Zhang, 2018). Polystely in the different species of *P.* sect. Litobrochia differ slightly, mostly in relation to the size and number of vascular cylinders, although this may vary with size and age of the individual. In general, *P. altisima* has two concentric solenosteles with the inner cylinder fusing with the outer one at each node or leaf gap (Bower, 1928). This is similar to what is observed in *P. muricata* (Fig. 1c). In contrast, polystely in species such as *P. lividia* and *P. podophylla*, shows the stele to be more elaborated and there may be three or more concentric cylinders (Fig. 1c). The elaboration of the stele in *P.* sect Litobrochia may be correlated with rhizome and leaf size (Bower, 1928).

DISCUSSION

The section *P.* Litobrochia is a strictly South American clade including 11 species (Zhang and Zhang, 2018). The character states that define this clade include reticulate venation, large fronds, and stout, short creeping to decumbent rhizomes (excluding *P. deflexa* and *P. muricata*) (Zhang and Zhang, 2018). Here, I describe a polycyclic solenostele as a new synapomorphy defining the section, furthering our understanding of the morphological circumscription of the genus.

The estimation of the ancestral states of stelar architecture by stochastic character mapping illustrates the evolution of stelar morphology in *Pteris*. The ancestral, and most common, stelar architecture in *Pteris* is a monocyclic solenostele with occasional transition to dictyosteley within an individual (Bower, 1928; Ogura, 1972). This transitionary stele arises from overlapping or failure of overlapping leaf gaps (Gwynne-Vaughan, 1903). Leaf gaps in a solenostele can overlap and become dictyostelic by either a reduction in the distance between leaves along an axis, *i.e.*, a reduction in internode length, or by an 'increase' in the length of the leaf gap itself. In *Pteris,* leaf gaps may be relatively short, as even in compact/erect rhizomes a solenostelic morphology is observed (Fig. 1e, f).

Based on the reconstructions, there are two origins of polystely in *Pteris*, one evolving in the most recent common ancestor of *Pteris* sect Litobrochia and the other on the branch leading to *P. wallichiana* (Fig. 2). It may be possible that other species related to *P. wallichiana*, including *P. tripartita, P. aborea, P. warburgii*, also possess a polycyclic solenostele, but more investigation is needed, and specimens of these species were not abundantly available to me. Specimens of *P. tripartite* examined were monocyclic, but again the available material for this species was limited. If this clade also possessed a polycyclic solenostele, it may represent another independent origin of this character state in the genus. While not included in the analysis due to confounding phylogenetic results (Zhang and Zhang, 2018), the ceratopteroid clade possesses a polycyclic stelar system (Thomas, 1905; Bower, 1928), which most likely represents independent evolution of polycycly in this clade.

The outgroups of *Pteris* sect. Litobrochia include *P.* sect Creticae, *P.* sect. Mutilatae, and *P.* sect. Dentatae all possess monocyclic solenosteles (Fig. 1d; Fig 2.). Stelar morphology seems to be relatively conserved phylogenetically, and when new stelar architectures arise they may serve well in taxonomic identification of certain clades. In addition to traits outlined in Zhang and Zhang (2018), polystely can be used as a consistent field character to narrow down species of *Pteris* to sect. Litobrochia when in the neotropics. However, care must be taken when dealing with young plants as immature stelar architecture may take on the form of a monocyclic solenostele or even a protostele in very young individuals (Bower, 1922; 1924).

The functional significance of polycycly has been speculated on by Tansley (1907a; b). He postulated that polycyclic vascular systems are selected for increasing vascular tissue leading to increased water supply to large leaved ferns. Most species in *Pteris* sect. Litobrochia are known to possess large leaves with reticulate venation, meaning they should have higher transpiration rates (Boyce *et al.*, 2009; Brodribb, 2009). However, there are other clades of *Pteris* that have large leaves and reticulate venation that do not have polycyclic solenosteles (such as *P.* sect. Denticulatae). Also, hydraulic conductivity scales to the fourth power of the radius of the conduit (Zimmermann, 1983), so increasing conduit dimensions as opposed to conduit number should have a larger effect on total hydraulic conductivity. An alternative hypothesis suggested by Bower (1922; 1924) is that there may be a correlation between rhizome size and concentric bundle number. During my investigation it was noticed that larger specimens possessed more elaborate steles. It seems likely that various stelar architectures–including polystely–arise through processes of shoot development such as phyllotaxy and rhizome diameter.

While character reconstructions demonstrate that there was an independent origin of polycycly in *Pteris* sect. Litobrochia, there may be other polycyclic species in *Pteris* not sampled here. Future studies that include more species, three dimensional reconstructions, and developmental series should be conducted to understand the evolution of stelar architecture in this genus at a deeper level. Specifically, species in the subclade *Tripartita* (Zhang and Zhang, 2018) may possess polycyclic steles and represent another independent evolution of this stelar morphology in the genus. Moreover, *Pteris* species that conform to the sect Litobrochia morphology, including large rhizomes, large leaves, and reticulate venation, should be investigated to determine if polycycly is more widespread and correlated with these traits. The genus *Pteris* has been notoriously difficult to circumscribe, and the new findings presented here throw light on the morphological circumscription and evolution of stelar architecture in this genus.

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SHORTER NOTES

Nesting Spores in a Cave: First Anatomical Characterization of *Gleichenia polypodioides* (Gleicheniaceae) Soral Chamber.—The taxonomy of Gleicheniaceae has undergone several changes recently (Gonzales and Kessler, Phytotaxa 31:1–54. 2011). The monophyly of some genera has not been elucidated yet due to poor sampling, and few phylogenetic studies have been completed to date (PPG I, Journal of Systematic and Evolution 54:563–603. 2016). Currently, seven genera are accepted in the family, including the paleotropical *Gleichenia* Sm. Initially, this genus was broadly circumscribed and included all species currently placed in *Diplopterygium* (Diels) Nakai, *Dicranopteris* Bernh, *Gleichenella* Ching, and *Sticherus* C. Presl (Gonzales and Kessler, 2011). However, molecular and morphological studies supported the segregation of those genera from *Gleichenia* (Copeland, Annales Cryptogamici et Phytopathologici 5:1–2471. 1947; Nakai, Bulletin of the National Science Museum 29: 1–71. 1950; Pryer *et al.*, American Journal of Botany 91:1582–1598. 2004; Li *et al.* Acta Palaeontologica Sinica 49:64–72. 2010). Additionally, another genus, *Rouxopteris* H.M. Liu, endemic to the Madagascar region, was recently segregated from *Gleichenia* based on *rbcl* sequences and morphological evidence (Liu *et al.,* Plant Systematics and Evolution 306:30. 2020). Currently, *Gleichenia* comprises about 10 species with paleotropical distribution, three of which occur in Africa, and the others are distributed through South East Asia, Malesia, Australia, New Caledonia, and New Zealand (Perrie *et al.*, New Zealand Journal of Botany 50:401-410. 2012). *Gleichenia* is characterized by buds and rhizomes covered with scales, pinnate-pinnatifid ultimate branches with reduced rounded segments, and tetrahedral, trilete spores. In addition, two species of *Gleichenia* have the sori arranged in deep depressions, a rare character among the ferns: the African *Gleichenia polypodioides* (L.) Sm. and *Gleichenia inclusisora* Perrie, L.D.Sheph. & Brownsey, from New Zealand (Perrie *et al.*, 2012). Sori in those species are usually composed of three sporangia, rarely four, enclosed in a deep depression (here referred to as a soral chamber), convex toward the abaxial leaf surface, with the sporangia stomia facing the chamber opening. Moreover, some other ferns have sori in depressions, such as *Lellingeria depressa* (C.Chr.) A.R.Sm. & R.C.Moran - Polypodiaceae (Labiak and Prado, Revista Brasileira de Botânica 28: 1–22. 2005) and *Amauropelta soridepressa* (Salino & V.A.O. Dittrich) Salino & T.E.Almeida -Thelypteridaceae (Salino and Dittrich, American Fern Journal 98: 199–201. 2008). However, these depressions are shallow and the sporangia are exposed on the leaf surface. Although the presence and general morphology of the soral chamber has been previously reported, not much is known about the anatomy and development. Here, we provide the first anatomical description of this unique sori morphology in the Gleicheniaceae. Regarding the Gleicheniaceae leaf terminology, we followed Andersen and Øllgaard (American Fern Journal 86: 52–57. 1996), with modifications. The anatomical studies were performed on sterile (Fig. 1A) and fertile ultimate branches of *Gleichenia polypodioides* (Fig. 1J) (BHCB 189155 - South Africa, Cape City, *M. Lima s.n.*). The sterile (n=3) and fertile branches (n=3) were hydrated in hot water, followed by a two-hour immersion in 2% potassium hydroxide, and 2-3 times washing to complete the reversion process (Smith and Smith, American Journal of Botany 29:464–471. 1942, modified). The branches were dehydrated in an ethanol series, before embedding in Historesin (Leica Microsystems GmbH, Wetzlar, Germany). Transverse sections (6-8 μm) were produced using a Reichert Jung Leica® biocut rotary microtome. The histological sections were stained with 0.05% toluidine blue in acetate buffer, pH 4.7 (O'Brien, Feder, and McCully, Protoplasma 59:368–373. 1964), and mounted on slides with water. All samples were analyzed and photographed on a light microscope (Leica DM500) with a coupled camera (Leica ICC50 HD). The sterile segments (Fig. 1A-B) are glabrous, hypostomatic with dorsiventral organization, and the margins of the segments are slightly revolute. The epidermis is uniseriate constituted of thick-walled periclinally elongated cells, covered by thin cuticle, both on the abaxial and adaxial surfaces (Fig. 1C-D). The abaxial epidermal cells are smaller than the adaxial epidermal cells, and at the midrib region, epidermal cell walls are lignified (Fig. 1E). The mesophyll has 1-2 layers of palisade-like parenchyma with polygonal juxtaposed cells, and 3-4 layers of spongy parenchyma (Fig. 1F-G). The vascular tissues in the midrib vein have an amphicribral arrangement and are surrounded by 4-5 layers of pericyclic fibers (Fig. 1H-I). On the fertile segment (Fig. 1J-K), the epidermis is uniseriate, and similar to the sterile segment on the adaxial surface. The unique aspect is the formation of the soral chamber in the fertile segment by the invagination of the blade tissue. Underlying the soral chamber on the abaxial surface, there is the epidermis and the 2-3 layered spongy parenchyma (Fig. 1L). The soral chamber usually bears two to three sporangia (Fig. 1J-K). The sporangia have a typical gleichenious leptosporangial morphology, with no morphological peculiarity. The base of the sporangia is connected to the secondary veins by a short multicellular stalk with polygonal cells (Fig. 1M-N). The annulus is oblique, with thick-walled cells and a discrete stomium, lined by thin-walled cells. The annulus cells are distal and thick-walled, while the stomium has thin-walled cells and usually face the soral chamber opening (Fig. 1O-P). Numerous trilete spores develop inside the chamber (Fig. 1O-P). The overall leaf morphology of *G. polypodioides* observed in the present study accords with previous anatomical studies performed in Gleicheniaceae, especially in *Gleichenia s.s.* (Boodle, Annals of Botany 15:703–747. 1901; Chrysler, American Journal of Botany 30: 735-74. 1943: Chrysler, American Journal of Botany 31:483–491. 1944; Ogura, Comparative anatomy of vegetative organs of the Pteridophytes, p. 336. 1972). It should be noted that all species of Gleicheniaceae, with the exception of *G. polypodioides* and *G. inclusisora*, have superficial sori. Therefore, the soral chamber represents a distinctive taxonomic feature, and a very useful character to readily recognize these two species. A third species, *G. dicarpa* var. *alpina* (R. Br.) Hook. f. [≡*Gleichenia alpina* R. Br.], has a chamber-

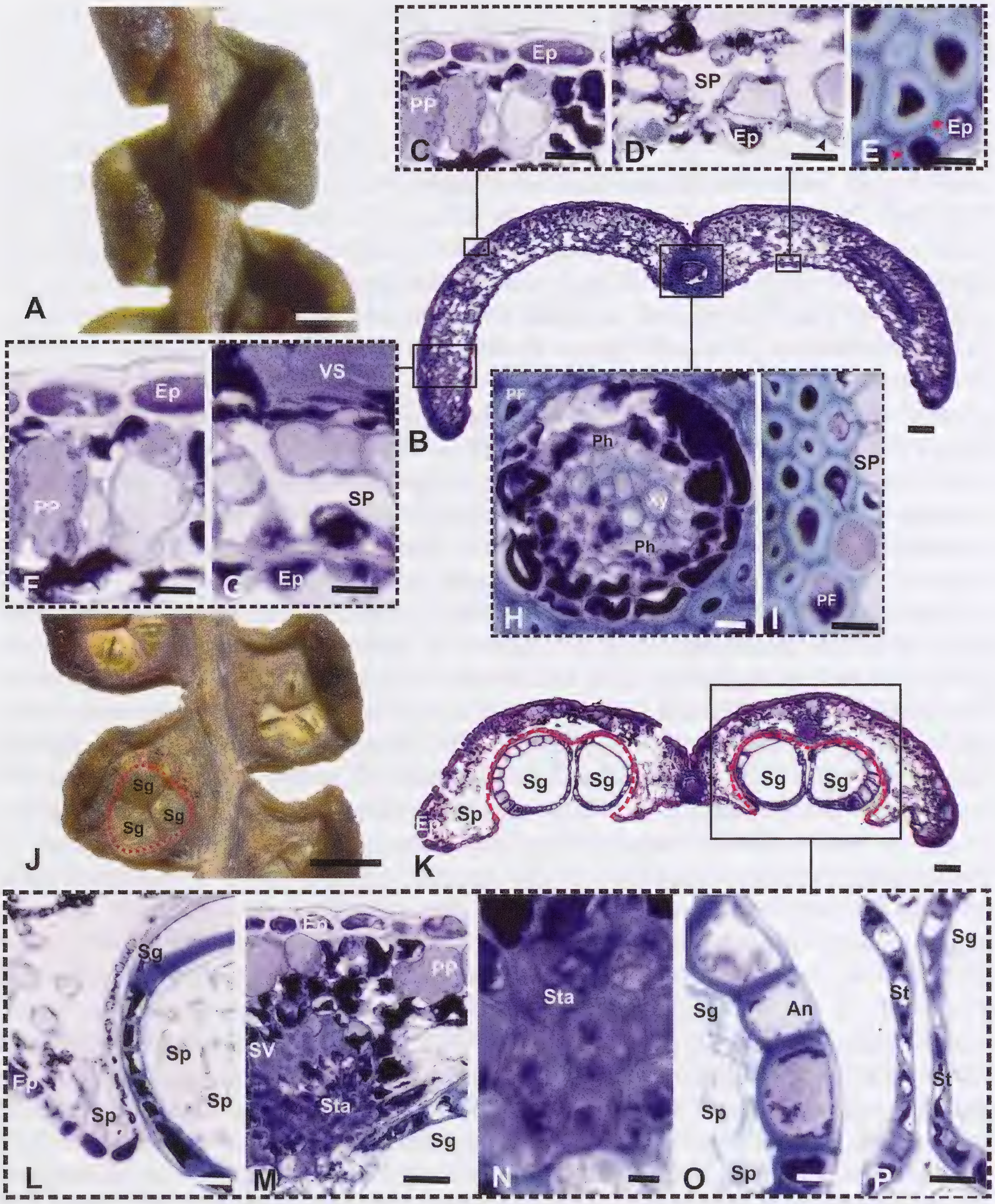

FIG. 1. Anatomy of sterile and fertile ultimate branches of *Gleichenia polypodioides*. A. General aspect of a sterile branch; B. General view of a transverse anatomical section of a sterile branch; C-E. Details of epidermal cells. C. Adaxial epidermal surface with periclinally elongated cells, and palisade parenchyma adjacent cells; D. Abaxial epidermal surface and spongy parenchyma adjacent cells; E. Epidermal and adjacent cortical cells at the midrib region with lignified walls (red arrow); F. Palisade-like parenchyma in detail; G. Spongy parenchyma cells in detail; H. Detail of the midrib vein showing phloem, xylem, and pericyclic fibers; I. Pericyclic fibers in detail; J. General aspect of a fertile branch showing soral chambers (red dotted), and sori with three sporangia; K. Transverse anatomical section of a fertile branch showing dermal and ground systems. The midvein is diminutive. Soral chamber with sori (limited by red dotted lines); L. Detail of the epidermis and

like organization of the soral area formed by the revolute margin of the ultimate segments and the scales borne around the sori. This chamber is dissimilar of those of *G. polypodioides* and *G. inclusisora*, but likely functions for protection of the sori (Boodle, 1901). The arrangement of the scales around the sori is rather common and observed in other groups of leptosporangiate ferns, such as in the species of *Pleopeltis* (Polypodiaceae). The presence of scales and trichomes is usually related to water loss control, as dense indument may act by reflecting the light, reducing the leaf temperature, and thus reducing transpiration (Hietz, Fern Ecology, p. 144. 2010). Some other Gleicheniaceae species have scales or trichomes across the abaxial surface, such as *Sticherus bifidus* (Wild.) Ching and *Sticherus lanuginosus* (Fée) Nakai, yet other species, such as *Sticherus brevitomentosus* Østergaard & Øllgaard, have scales restricted to the soral area. The soral chamber seems to be an adaptation of the fertile portion of the blade for protection of the sori and drought avoidance, and may be ontogenetically related with sporangial development. Drought avoidance strategies favor Gleicheniaceae species, which are usually sunny plants and occupy open and disturbed habitats and forests edges (Gonzales and Kessler, 2011; Lima and Salino, Phytotaxa 358:199–234. 2018). Such areas are characterized by higher light levels and lower relative humidity than the understory (Hietz, 2010). Additionally, stomata on the abaxial surface (Ogura, 1972) associated with the abaxial indument may play a role in drought avoidance for Gleicheniaceae species. However, *G. polypodioides* plants have a glabrous abaxial leaf surface, and the soral chamber may act as an alternative adaptation to avoid desiccation, reduce water loss, and protect against the excessive light radiation. Regarding *G. inclusisora*, besides the soral chamber, some sparse scales and a white farina on the abaxial surface of leaflet segments (Perrie and Brownsey, Flora of New Zealand: ferns and lycophytes. Gleicheniaceae p. 22. 2015) may also be related to drought avoidance and soral protection. Further anatomical and ontogenetic studies of *Gleichenia inclusisora* may elucidate the origin and development of its soral chamber. Additionally, phylogenetic studies with morphological character optimization may help to reveal the evolution of the soral chambers in *Gleichenia*.

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spongy parenchyma underlying the soral chamber; M. Detail of the insertion of the sporangia to the secondary vein. N. Detail of cells of the multicellular stalk with conspicuous nucleus and dense cytoplasm. O. Detail of the annulus; P. Detail of the stomium. An. Annulus; Ep. Epidermis; PF Pericyclic fibers; Ph. Phloem; PP. Palisade-like parenchyma; SP. Spongy parenchyma; Sg. sporangia; Sp. Spore; Sta. Stalk; St. Stomium; VS. Vascular system; Xy. Xylem. Bars: A and J 1cm; Bars: B and K 200 μm; Bars: C, D, E, F, G, I, L, M, O and P 50 μm; Bars: H and N 20 μm.

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COVER CAPTION: *Diplazium molokaiense.* Photo credit: Hank Oppenheimer